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Attorney Docket P1101P2
PATENT

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by [initials]

CERTIFICATION UNDER 37 CFR 1.10

EL141796136 US Express Mail Number September 15, 1999 Date of Deposit

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Ann Savelli
Ann Savelli

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

09/15/99
JCS96 U.S. PTO
09/15/99

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Avi J. Ashkenazi
Anan Chuntharapai
K. Jin Kim

Title: **APO-2 RECEPTOR ANTIBODIES**

1. Type of Application

This application is for an original, non-provisional application.

This is a non-provisional application claiming priority to provisional application no. __, filed __, the entire disclosure of which is hereby incorporated by reference.

This is a continuation-in-part continuation divisional application claiming priority to application Serial Number 09/096,637, filed June 12, 1998, and Serial Number 09/020,746, filed February 8, 1998, the entire disclosures of which are hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b)
(Non-provisional)**

87 pages of specification
2 pages of claims
1 page(s) of abstract
22 sheet(s) of drawings
[] formal informal

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)

An executed declaration of the inventor(s) [] is enclosed [x] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)

A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)

A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)

An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [x] will follow.

(for cont./div.)

The prior application is assigned of record to Genentech, Inc.

5. Amendments *(for continuation and divisional applications)*

Cancel in this application original claims ___ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

Amend the specification by inserting before the first line the sentence:
--This is a

non-provisional application

continuation

divisional

continuation-in-part

of co-pending application(s)

Serial No. __ filed on __, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --

International Application __ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

provisional application No. __ filed __, the entire disclosure of which is hereby incorporated by

reference and to which application(s) priority is claimed under 35 USC §119--.

6. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$760.00
Total Claims	13	- 20 =	0	X \$18.00	\$0.00
Independent Claims	2	- 3 =	0	X \$78.00	\$0.00
— Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$760.00

7. Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$760.00. A duplicate copy of this transmittal is enclosed.

8. Authorization to Charge Additional Fees

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. A duplicate copy of this sheet is enclosed.

9. Additional Papers Enclosed

- Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- A new Power of Attorney or authorization of agent.
- Other:

10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

[This item must be completed and the necessary papers filed in the prior application if the period set in the prior application has run]

- _____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
- _____ A copy of the petition for extension of time in the **prior** application is attached.

11. Correspondence Address:

X Address all future communications to:

GENENTECH, INC.
Attn: Diane L. Marschang
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-1000

Respectfully submitted,
GENENTECH, INC.

Date: September 15, 1999

By: Diane L. Marschang
Diane L. Marschang
Reg. No. 35,600

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1000
Fax: (650) 952-9881

10

Apo-2 Receptor AntibodiesRELATED APPLICATIONS

15 This application is a continuation-in-part application of Application Serial No.09/096,637 filed June 12, 1998 and a continuation-in-part application of Application Serial No. 09/020,746 filed February 8, 1998, which is a continuation-in-part application of Application Serial No. 08/857,216 filed May 15, 1997, the contents of which are hereby incorporated by reference.

20

FIELD OF THE INVENTION

25 The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2" and to anti-Apo-2 antibodies.

29

BACKGROUND OF THE INVENTIONApoptosis or "Programmed Cell Death"

30 Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell 35 death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. 40 Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, 45 Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia,

5 myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, 10 segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; 15 Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified 20 oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been 25 observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- α ("TNF- α "), tumor necrosis factor- β ("TNF- β " or "lymphotoxin"), CD30 ligand, CD27 25 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, 30 Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633 published January 16, 1997]. Among these molecules, TNF- α , TNF- β , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been 35 reported to be involved in apoptotic cell death. Both TNF- α and TNF- β have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- α 40 is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on

5 Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the 10 periphery [Krammer et al., *Curr. Op. Immunol.*, 6:279-289 (1994); Nagata et al., *Science*, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated 15 lymphocytes when their function is no longer needed [Krammer et al., *supra*; Nagata et al., *supra*]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- α [Yonehara et al., *J. Exp. Med.*, 169:1747-1756 (1989)].

TNF Family of Receptors

20 Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., *J. Biol. Chem.*, 264:14927-14934 (1989); Brockhaus et al., *Proc. Natl. Acad. Sci.*, 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been 25 isolated and characterized [Loetscher et al., *Cell*, 61:351 (1990); Schall et al., *Cell*, 61:361 (1990); Smith et al., *Science*, 248:1019-1023 (1990); Lewis et al., *Proc. Natl. Acad. Sci.*, 88:2830-2834 (1991); 30 Goodwin et al., *Mol. Cell. Biol.*, 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., *Immunogenetics*, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The 35 extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., *EMBO J.*, 9:3269 (1990); and Kohno, T. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 87:8331 (1990)]. The cloning of recombinant soluble TNF receptors was reported by Hale et al. [*J. Cell. Biochem. Supplement* 15F, 1991, p. 113 (P424)].

40 The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four

5 cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH₂-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al.,
supra; Loetscher et al., supra; Smith et al., supra; Nophar et al.,
supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of
10 the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2-
amino acids from about 54 to about 97; CRD3- amino acids from about 98
to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2,
CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about
15 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4-
amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435
(1993)]. The potential role of the CRDs in ligand binding is also
described by Banner et al., supra.

20 A similar repetitive pattern of CRDs exists in several other
cell-surface proteins, including the p75 nerve growth factor receptor
(NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature,
325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J.,
8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063
(1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al.,
supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2
25 proteins of the Shope and myxoma poxviruses [Upton et al., Virology,
160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335
(1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of
these sequences indicates that the positions of the cysteine residues
are well conserved. These receptors are sometimes collectively
30 referred to as members of the TNF/NGF receptor superfamily. Recent
studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et
al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid
insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem.,
266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H.
35 and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of
about 60 amino acids, between its CRD4 and transmembrane region, which
is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol.,
41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973
(1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region
40 is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an
apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh

5 et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- α or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 10 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- α , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) 15 family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- α , Apo-1 20 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble 25 receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), 30 investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member 35 referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

The Apoptosis-Inducing Signaling Complex

40 As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors;

5 in *C. elegans*, these elements are encoded respectively by three genes, Ced-4, Ced-9 and Ced-3 [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 10 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-KB [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et 15 al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleavland and Ihle, Cell, 81:479-482 (1995)].

20 Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD 25 serves as an adaptor protein which recruits the Ced-3-related protease, MACH α /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. Caspase 8 appears to be the trigger that sets off a cascade of apoptotic 30 proteases, including the interleukin-1 β converting enzyme (ICE; caspase 1) and CPP32/Yama (caspase 3), which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

35 It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, ced-3, and to the mammalian IL-1-converting enzyme, ICE (caspase 1). The activity of caspase 1 and 3 can be inhibited by the product of the cowpox virus gene, crmA [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell 40 death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

5 As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-KB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-KB is the prototype of a family of 10 dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the IKB in response to certain stimuli, released NF-KB translocates to 15 the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

20 SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF-KB. Applicants surprisingly found that the soluble 30 extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can inhibit Apo-2 ligand function. The crystal structure of the complex formed between Apo-2 ligand and an extracellular domain sequence of Apo-2 is described herein. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described 35 Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). 40 In other embodiments, the isolated Apo-2 polypeptide comprises at least

5 about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

10 In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

15 In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

20 In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

25 In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

30 (a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

35 (b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

40 (c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e.,

5 nucleotides 1109-1111 through 1310-1312), inclusive; or
10 (e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code. The isolated nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the vector deposited as ATCC 209021 which includes the nucleotide sequence 10 encoding Apo-2 polypeptide.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is 15 further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic, neutralizing, or blocking antibody. Dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also 20 provided.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence 25 human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native 30 sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death 35 domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells 40 transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated

5 proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based 10 plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 15 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF-KB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by 20 an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-KB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor 30 expressed in human 9D cells.

Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

Figure 9 is a bar diagram showing percent (%) apoptosis, as 35 compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L in 9D cells.

Figure 11 is a graph showing results of an ELISA testing 40 binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.

5 Figure 12 provides a table identifying Apo-2 antibodies, 2B3.7.1 ("2B3"); 3F11.39.7 ("3F11"); 4B9.23.6 ("4B9"); 5C7.9.1 ("5C7"); 3H1.18.10 ("3H1"); 3H3.14.5 ("3H3"); 3D5.1.10 ("3D5"); 3C9.8.6 ("3C9"); 4H10.14.10 ("4H10"), as well as various properties and activities identified with each respective antibody.

10 Figure 13 shows the binding affinities of Apo-2 antibodies 3F11 and 3H3, to Apo-2-IgG, as determined in a KinExA™ assay. Binding affinities, e.g., of DR4 and Apo-2 immunoadhesins to Apo-2 ligand are shown for comparison.

Figure 14 shows apoptotic activity of Apo-2 antibodies, 3D5; 15 3C9; 5C7; 3H3; 3F11; 4B9; 2B3; and 3H1, on SKMES tumor cells in the presence of goat anti-mouse IgG Fc.

Figure 15A shows apoptotic activity of Apo-2 antibodies, 3F11 and 3H3, on Colo205 tumor cells in the presence or absence of goat anti-mouse IgG Fc.

Figure 15B shows apoptotic activity of Apo-2 antibodies, 3F11 and 3H3, on glioma cells in the presence or absence of goat anti-mouse IgG Fc.

Figure 16 shows graphs illustrating results of an ELISA testing binding of Apo-2 antibodies, 3D5; 3F11; 3H1; and 3H3, to Apo-2 and to other known Apo-2 ligand receptors referred to as DR4 and DcR1.

Figure 17 shows the results of an ELISA testing for blocking activity of Apo-2 antibodies, 3F11; 3H3; 2B3; 4B9; 5C7; 3H1; 3D5; 3C9; and 4H10.

Figure 18 shows the effects of Apo-2 antibodies, 3F11 and 3H3, on the growth of Colo205 tumors in athymic nude mice, as measured by tumor volume.

Figure 19 shows data collection and refinement statistics for the x-ray crystal structure of Apo-2 (DR5) bound to Apo-2 ligand.

Figure 20 shows the Apo-2L/Apo-2 complex. The Apo-2L trimer is drawn as ribbon and the three receptors are rendered as tubes. The disordered loop (residues 132-143) in Apo-2L is rendered as small spheres. The bound zinc atom and the bound chloride ion pink are shown as small spheres. Beta strands and relevant loops are labeled. (A) Side view. In this orientation, the membrane of the receptor-containing cell is at the bottom of the figure. (B) View down the three-fold axis of the complex, perpendicular to (A).

Figure 21 shows an open-book view of the Apo-2L/Apo-2

5 interface. Apo-2L and one receptor are rendered as space filling models, while the other two receptors are shown as tubes. Residues in the interface are colored by % of buried accessible surface area upon complex formation (1-25%, very light grey; 25-50%, light grey; 50-75%, grey; 75-100%, dark grey). The interface divides into two patches, A
10 and B (labeled). A probe size of 1.4 Angstrom was used to calculate the accessible surface area.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

15 The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by
20 recombinant or synthetic methods.

25 A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of
30 the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino
35 acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence
40 Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a

5 form of Apo-2 which is essentially free of the transmembrane and
cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than
1% of such transmembrane and/or cytoplasmic domains and preferably, will
have less than 0.5% of such domains. Optionally, Apo-2 ECD will
comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino
10 acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1). The Apo-2 extracellular
domain sequence may also comprise amino acid residues 1 to 130 of Fig. 1
(SEQ ID NO:1).

15 "Apo-2 variant" means a biologically active Apo-2 as defined
below having at least about 80% amino acid sequence identity with the
Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID
NO:1) for a full-length native sequence human Apo-2 or with the deduced
amino acid sequences for an extracellular domain or death domain of Apo-
2 identified herein. Such Apo-2 variants include, for instance, Apo-2
20 polypeptides wherein one or more amino acid residues are added, or
deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID
NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino
acid sequence identity, more preferably at least about 90% amino acid
sequence identity, and even more preferably at least about 95% amino
acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID
NO:1) or with the extracellular domain or death domain sequences of Apo-
2.

25 "Percent (%) amino acid sequence identity" with respect to
the sequences identified herein is defined as the percentage of amino
acid residues in a candidate sequence that are identical with the
amino acid residues in the Apo-2 sequence, after aligning the
30 sequences and introducing gaps, if necessary, to achieve the maximum
percent sequence identity, and not considering any conservative
substitutions as part of the sequence identity. Alignment for
purposes of determining percent amino acid sequence identity can be
35 achieved in various ways that are within the skill in the art can
determine appropriate parameters for measuring alignment, including
assigning algorithms needed to achieve maximal alignment over the
full-length sequences being compared. For purposes herein, percent
amino acid identity values can be obtained using the sequence
40 comparison computer program, ALIGN-2, which was authored by Genentech,
Inc. and the source code of which has been filed with user
documentation in the US Copyright Office, Washington, DC, 20559,

5 registered under the US Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, CA. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

10 The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the 15 antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

20 "Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous 25 or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, 30 silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

35 An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are 40 distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily

5 express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for 10 prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, 15 DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. 20 Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. 25 Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 30 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may 35 be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a 40 single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including

5 hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv),
10 so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

15 Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

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40 "Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of

5 the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

10 The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I¹³¹, I¹²⁵, Y⁹⁰ and Re¹⁸⁶), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or variants and/or fragments thereof.

15 A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, NJ), and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, France), toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

20 30 "Biologically active" and "desired biological activity" with respect to an Apo-2 polypeptide for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and 35 Apo-2 ligand activity.

40 The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and

5 measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

10 The terms "cancer", "cancerous" or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous
15 cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioma, neuroblastoma, cervical cancer, ovarian cancer, lung cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, glioblastoma, endometrial carcinoma, salivary gland carcinoma, kidney cancer, colorectal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that
35 Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2 and Apo-2 variants, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

40 A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing

5 Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

10 The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained 15 from a genomic library or by oligonucleotide synthesis.

19 Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

20 A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of 25 sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P -labeled ATP, biotinylation or enzyme labeling. 30 Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

35 Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described 40 in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into

5 cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such 10 as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-15 conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one 20 amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis 25 [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques 30 can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify 35 one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it 40 eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be

5 contacted with, for instance, Apo-2L, and the interaction, if any, can
be determined. The interaction between the Apo-2 variant and Apo-2L can
be measured by an *in vitro* assay, such as described in the Examples
below. While any number of analytical measurements can be used to
compare activities and properties between a native sequence Apo-2 and an
10 Apo-2 variant, a convenient one for binding is the dissociation constant
 K_d of the complex formed between the Apo-2 variant and Apo-2L as
compared to the K_d for the native sequence Apo-2. Generally, a \geq 3-fold
increase or decrease in K_d per substituted residue indicates that the
substituted residue(s) is active in the interaction of the native
15 sequence Apo-2 with the Apo-2L.

20 Optionally, representative sites in the Apo-2 sequence
suitable for mutagenesis would include sites within the extracellular
domain, and particularly, within one or both of the cysteine-rich
domains. Such variations can be accomplished using the methods
described above.

25 As described in the Examples below, the crystal structure of
the complex between Apo-2 ligand and an extracellular domain sequence of
Apo-2 receptor has been determined. Apo-2 resembles TNFR1 in overall
structure with relatively little defined secondary structure. It is
tethered into an elongated shape by a series of seven disulfide bridges,
30 six of which are found in subdomains of Apo-2 (residues 43-84 and 85-
130, respectively; see Fig. 1, SEQ ID NO:1) that correspond structurally
to the second and third CRDs of the TNFR1 receptor. The first disulfide
bridge of Apo-2, between residues 28 and 41 (Fig. 1; SEQ ID NO:1),
35 corresponds to the last disulfide bridge (between Cys 33 and Cys 52) in
CRD1 of TNFR1, while the first 21 residues of Apo-2 are disordered.
Thus, Apo-2 residues 1-42, 43-85, and 86-130 (Fig. 1; SEQ ID NO:1) form
40 analogous subdomains to CRD1, CRD2 and CRD3 of TNFR1. The three copies
of Apo-2 in the complex are very similar to each other, with the
exception of the C-terminal portion of CRD3 (residues 104-130; Fig. 1;
SEQ ID NO:1), which exhibits a rigid-body variation in orientation. The
two loops that form most of the contacts with the ligand have very
similar conformations in all three copies.

45 Unlike the TNF-beta-TNFR1 complex, where the C-terminal
subdomain of the receptor was disordered, the C-terminal residues of
Apo-2 are well ordered up to residue 128 in one copy (the "R" chain)
and up to residue 130 in the other two (the "S" and "T" chains).

5 Residue 130 is predicted to be the final extracellular residue before the putative single transmembrane helix connecting the receptor to its intracellular death domain. In the complex as described in Example 21 below, the C-termini of the receptors form a triangle approximately 50 Å on a side. Intriguingly, this same spacing is also found for the
10 receptor binding sites on the TRAF-2 trimer, which is known to interact with the intracellular portions of some TNFR family members (albeit not with DR4 or Apo-2) (Park et al., *Nature*, 398:533-538 (1999); McWhirter et al., *Proc. Natl. Acad. Sci.*, 96:8408-8413 (1999)). This suggests that the observed extracellular geometry could
15 be propagated through the rigid transmembrane helix to the death domains, and that this spacing may be important for proper triggering of the intracellular apoptotic cascade.

The interface of the Apo-2 ligand/Apo-2 complex is divided into two patches- patch A and patch B. The dominant characteristic of patch B in the Apo-2L/Apo-2 interface is the interaction between Tyr 216 of Apo-2L (using the numbering of the amino acid sequence for Apo-2L provided in Pitti et al., *J. Biol. Chem.*, 271:12687-12690 (1996)) and the 50s loop of the Apo-2 receptor. Residue Tyr 216 is conserved in many of the TNF superfamily ligands (including TNF-alpha, TNF-beta, FasL and OPGL), while other members have a similar large hydrophobic residue at this position. Mutagenesis studies on TNF-alpha, TNF-beta, FasL and Apo-2L have all shown that this residue is critical for binding (Schneider et al., *J. Biol. Chem.*, 272:18827-18833 (1997); Goh et al., *Protein Eng.*, 4:785-791 (1991); Yamagishi et al., *Protein Eng.*, 3:713-719 (1990); Van Ostdae et al., *Protein Eng.*, 7:5-22 (1990); Hymowitz et al., personal communication). The interactions of the tyrosine side chain are conserved between the Apo-2L/Apo-2 and TNF-beta-TNFR1 complexes. Moreover, the backbone conformation of the 50s loop of the receptor, which forms the binding pocket for the side
20 chain, is virtually identical between Apo-2 and the TNFR1 (rmsd of only 0.35 between the C-alpha atoms of residues 51 to 62). Additionally, the length of this loop is conserved among the different TNF receptor superfamily members. It is believed that this loop may function as a general hydrophobic binding patch interacting with
25 conserved hydrophobic features on the ligand which may help properly orient the upper part of the receptor for more specific contacts mediated by CRD3.

5 In contrast to the conserved interactions in patch B, patch
A near the bottom of the interface involves interactions made by the
90s loop on CRD3 of Apo-2, which has a completely different
conformation than the corresponding loop in the TNFR1.

10 In patch B, it is believed that the 50s loop of the receptor
and Apo-2 ligand residue 216 provide a hydrophobic patch generally
important for binding, whereas in patch A, the receptor 90s loop and
the Apo-2 ligand residue at or near position 205 control the
specificity and cross-reactivity. Based on the structure identified
15 by Applicants, the 50s loop and the 90s loop of the Apo-2 receptor are
expected to carry most of the ligand-binding determinants.

20 Optionally, the Apo-2 variants of the invention will retain or possess
native sequence amino acid residues (as provided in Figure 1) in the
50s and/or 90s loop. It is contemplated that Apo-2 may be mutated (by
insertion, deletion, and/or substitution) in certain aspects which
25 will affect ligand binding. The histidine and phenylalanine residues
at positions 53 and 59, respectively, of the Apo-2 sequence are both
relatively large residues. These two residues are believed to contact
residues Asp218 and Ser159 of the Apo-2 ligand; thus introducing
larger side chains at the 53 and 59 positions of the Apo-2 sequence
30 may adversely affect Apo-2L affinity for Apo-2 (but improve affinity
for DR4). Similar mutations may be accomplished at positions 62, 65,
and/or 104 of the Apo-2 sequence. Mutations in any one or all of the
Apo-2 residues at positions 50 through 62 may affect binding of amino
acid residues at or near residue Tyr216 of the Apo-2L sequence, while
35 mutations in any one or all of the Apo-2 residues at positions 65
through 69, 90 through 104, 108, 111, or 122 may affect binding of
amino acid residues at or near residue Gln205 of Apo-2 ligand. It is
contemplated that mutations at such residue(s) may enhance or decrease
interactions (such as binding) with Apo-2 ligand.

35 2. Insertion of Nucleic Acid into A Replicable Vector

40 The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2
may be inserted into a replicable vector for further cloning
(amplification of the DNA) or for expression. Various vectors are
publicly available. The vector components generally include, but are
not limited to, one or more of the following: a signal sequence, an
origin of replication, one or more marker genes, an enhancer element, a
promoter, and a transcription termination sequence, each of which is

5 described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage 10 site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is 15 recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor 20 leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native 25 Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

30 The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid 35 sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 40 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma,

5 adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

10 Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

15 DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

20 25 30 35 40 (iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

35 One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes

5 under eukaryotic control to convey resistance to the appropriate drug
G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin,
respectively.

Another example of suitable selectable markers for mammalian
10 cells are those that enable the identification of cells competent to
take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The
mammalian cell transformants are placed under selection pressure that
only the transformants are uniquely adapted to survive by virtue of
15 having taken up the marker. Selection pressure is imposed by culturing
the transformants under conditions in which the concentration of
selection agent in the medium is successively changed, thereby leading
to amplification of both the selection gene and the DNA that encodes
Apo-2. Amplification is the process by which genes in greater demand
20 for the production of a protein critical for growth are reiterated in
tandem within the chromosomes of successive generations of recombinant
cells. Increased quantities of Apo-2 are synthesized from the amplified
DNA. Other examples of amplifiable genes include metallothionein-I and
-II, adenosine deaminase, and ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be
identified by culturing all of the transformants in a culture medium
25 that contains methotrexate (Mtx), a competitive antagonist of DHFR. An
appropriate host cell when wild-type DHFR is employed is the Chinese
hamster ovary (CHO) cell line deficient in DHFR activity, prepared and
propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA,
30 77:4216 (1980). The transformed cells are then exposed to increased
levels of methotrexate. This leads to the synthesis of multiple copies
of the DHFR gene, and, concomitantly, multiple copies of other DNA
comprising the expression vectors, such as the DNA encoding Apo-2. This
amplification technique can be used with any otherwise suitable host,
e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous
35 DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx
is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that
contain endogenous DHFR) transformed or co-transformed with DNA
40 sequences encoding Apo-2, wild-type DHFR protein, and another selectable
marker such as aminoglycoside 3'-phosphotransferase (APH) can be
selected by cell growth in medium containing a selection agent for the
selectable marker such as an aminoglycosidic antibiotic, e.g.,

5 kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., *Nature*, 282:39 (1979); Kingsman et al., *Gene*, 7:141 (1979); Tschemper et al., *Gene*, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant 10 strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, *Genetics*, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 15 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., *Curr. Genet.*, 12:185 (1987)]. More recently, an expression system 20 for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, *Bio/Technology*, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed 25 [Fleer et al., *Bio/Technology*, 9:968-975 (1991)].

25 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences 30 located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. 35 Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These 40 promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the

5 native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a 10 tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker 15 operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

20 Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription 25 of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

30 Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 35 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

40 Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytchrome C, acid phosphatase, degradative enzymes associated with

5 nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

10 Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus 15 and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

20 The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate 25 early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 30 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 35 (1982) on expression of the human interferon gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long 40 terminal repeat as a promoter].

5 (v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its 10 transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself 15 [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 20 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also 25 Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

25 (vi) Transcription Termination Component

30 Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These 35 regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

35 (vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated 40 in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12

5 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in Enzymology, 65:499 (1980).

10 (viii) Transient Expression Vectors

15 Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

20 (ix) Suitable Exemplary Vertebrate Cell Vectors

25 Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

30 3. Selection and Transformation of Host Cells

35 Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 40 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as

5 filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

10 Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and 15 insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 20 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

25 Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 30 35 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

35 Propagation of vertebrate cells in culture (tissue culture) 40 is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40

5 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); 10 mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

15 Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

20 Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the 25 operation of this vector occurs within the host cell.

30 Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with 35 *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

40 For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host

5 system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear
10 microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

15 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

20 The mammalian host cells used to produce Apo-2 may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

25 In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

30 The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

35 5. Detecting Gene Amplification/Expression

40 Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting,

5 Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ³²P. However, other techniques
10 may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific
15 duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

20 Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

25 30 Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

35 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

40 When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant

5 cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable 10 purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants 15 such as IgG.

20 Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of 25 affinity matrices also can be used.

30 A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is 35 introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for 40 crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful

5 for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional
10 imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light.
15 Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

20 Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular
25 Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

30 Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not
35 present in the native sequence Apo-2.

40 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain.

5 Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglucosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

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Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge

5 et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

10 Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

15 Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

8. Apo-2 Chimeras

20 The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

25 In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds 30 to the epitope tag.

35 Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 40 255:192-194 (1992)]; an α -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide

5 tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397
(1990)]. Once the tag polypeptide has been selected, an antibody
thereto can be generated using the techniques disclosed herein.

10 Generally, epitope-tagged Apo-2 may be constructed and
produced according to the methods described above. Epitope-tagged Apo-2
is also described in the Examples below. Apo-2-tag polypeptide fusions
are preferably constructed by fusing the cDNA sequence encoding the Apo-
2 portion in-frame to the tag polypeptide DNA sequence and expressing
the resultant DNA fusion construct in appropriate host cells.

15 Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the
present invention, nucleic acid encoding the Apo-2 will be fused at its
3' end to nucleic acid encoding the N-terminus of the tag polypeptide,
however 5' fusions are also possible. For example, a polyhistidine
sequence of about 5 to about 10 histidine residues may be fused at the
N- terminus or the C- terminus and used as a purification handle in
affinity chromatography.

20 25 30 Epitope-tagged Apo-2 can be purified by affinity
chromatography using the anti-tag antibody. The matrix to which the
affinity antibody is attached may include, for instance, agarose,
controlled pore glass or poly(styrenedivinyl)benzene. The epitope-
tagged Apo-2 can then be eluted from the affinity column using
techniques known in the art.

35 In another embodiment, the chimeric molecule comprises an
Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric
molecule may also comprise a particular domain sequence of Apo-2, such
as the extracellular domain sequence of native Apo-2 fused to an
immunoglobulin sequence. This includes chimeras in monomeric, homo- or
heteromultimeric, and particularly homo- or heterodimeric, or -
tetrameric forms; optionally, the chimeras may be in dimeric forms or
homodimeric heavy chain forms. Generally, these assembled
immunoglobulins will have known unit structures as represented by the
following diagrams.

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X or A

\ _____ C_H or C_L

X or A

10

\ _____ Y _____ C_H or C_L

A

A \ _____ C_L

\ _____ C_H

15

A

V_H \ _____ C_L

\ _____ C_H

20

V_L

A \ _____ C_L

\ _____ C_H

25

X

A \ _____ C_L

\ _____ C_H

30

A

X \ _____ C_L

\ _____ C_H

35

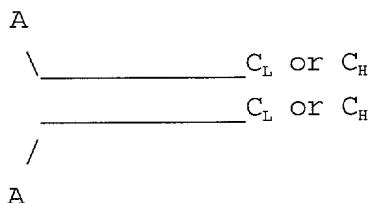
A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of 40 basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same

5 or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

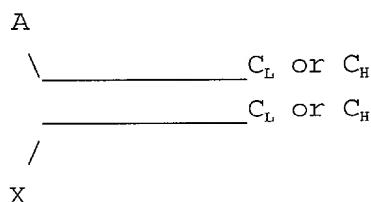
10 monomer: A _____ C_L or C_H

homodimer:



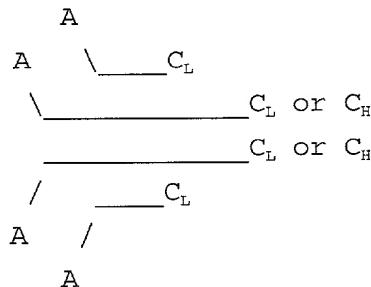
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heterodimer:



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homotetramer:

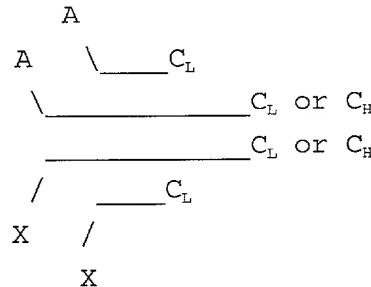


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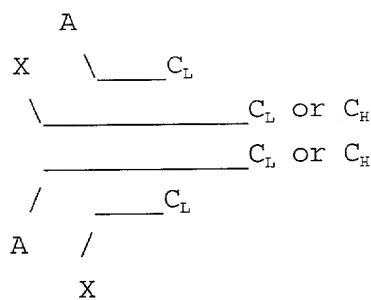
35

heterotetramer:



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5 and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- γ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and V_L , V_H , C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

40 In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of

5 another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et 10 al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

15 Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is 20 readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* 25 therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-30 288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

35 Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989. Molecules comprising the extracellular portion of CD4, the receptor for human 40 immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P

5 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991,
p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

10 Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene 15 therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand 20 that Apo-2 binds to.

25 The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, 30 procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

35 The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity 40 purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

45 Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of 40 therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a

5 prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic
10 sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2
15 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

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Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced

5 DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, 10 Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the 15 animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

20 **C. Anti-Apo-2 Antibody Preparation**

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

25 **1. Polyclonal Antibodies**

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein. 30 Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean 35 trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants 40 which may be employed include Freund's complete adjuvant and MPL-TDM

5 adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

10 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is 15 typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if 25 cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) 30 pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of 35 the unfused, immortalized cells. For example, if the parental transformed cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

40 Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as

5 HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of
10 human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

15 The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).
20 Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

25 After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

30 The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

35 The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The 40 hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells,

5 Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine
10 sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable
15 domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. Several of these antibodies have been deposited with ATCC, as disclosed herein. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456, HB-12535, HB-12534, or HB-12536. The term "biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. Particular activities and properties of various anti-Apo-2 antibodies are described in further detail in the Examples below. To determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, for instance, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below. The monoclonal antibody preferably has the hypervariable region residues of one or more of the above-mentioned ATCC deposited antibodies, e.g., it may comprise a humanized variant.
35 Chimeric and humanized Apo-2 antibodies derived from, constructed from, or containing sequence, regions or domains from any of the above-mentioned deposited antibodies are considered within the scope of the invention. Such chimeric or humanized Apo-2 antibodies may be prepared using techniques known in the art and further described below.

40 The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of

5 immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

10 *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348
15 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

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25 The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

3. Humanized Antibodies

35 The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues
40 from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human

5 immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, 10 in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human 15 immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et 20 al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534- 25 1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable 30 domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, 35 to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the 40 human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the

5 consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

10 It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three 15 dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

20 30 Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); 35 Bruggemann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et 40 al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, 10 and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light- 15 chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH₂, and CH₃ regions. It is preferred to have the first heavy-chain constant region (CH₁) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great 25 flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three 30 polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred 40 embodiment of this approach, the bispecific antibodies are composed of a

5 hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as
10 the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

15 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptopbutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

20 6. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., FEBS Letters, 30 409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433 (1997).

25 7. Conjugates

The invention also pertains to immunoconjugates comprising 35 Apo-2 antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate). Chemotherapeutic agents useful in the generation of such immunoconjugates have been 40 described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas*

5 *aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the trichothecenes.

10 A variety of radionuclides are available for the production of radioconjugate antibodies. Examples include ^{212}Bi , ^{131}I , ^{131}In , ^{90}Y and ^{186}Re .

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-15 succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science, 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene 20 triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

25 In another embodiment, the antibody may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the mammal, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

30 Immunoliposomes comprising Apo-2 antibody may also be prepared. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 35 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse 40 phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through

5 filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al.* J. Biol. Chem. 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained
10 within the liposome. See Gabizon *et al.* J. National Cancer Inst. 81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see
15 WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

20 Enzymes that are useful in this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents;
25 carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively,
30 antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs [see, e.g., Massey, Nature 328: 457-458 (1987)].
35 Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

5 The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen-binding region of an antibody of the invention linked to at least a 10 functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art [see, e.g., Neuberger et al., Nature, 312: 604-608 (1984)].

10 In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase 15 tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life.

15 This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation 20 of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

20 The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two 25 loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region 30 (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment. See, e.g., U.S. Patent No. 5,747,035.

35 Covalent modifications of the antibody are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody 40 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

45 The antibodies may optionally be covalently attached or conjugated to one or more chemical groups. A polyol, for example, can be

5 conjugated to an antibody molecule at one or more amino acid residues, including lysine residues as disclosed in WO 93/00109. Optionally, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), however, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene 10 glycol copolymers, can be employed using techniques for conjugating PEG to polypeptides. A variety of methods for pegylating polypeptides have been described. See, e.g. U.S. Patent No. 4,179,337 which discloses the conjugation of a number of hormones and enzymes to PEG and polypropylene glycol to produce physiologically active compositions having reduced 15 immunogenicities.

The antibodies may also be fused or linked to another heterologous polypeptide or amino acid sequence such as an epitope tag.

8. Other Modifications

Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies. For instance, cysteine residue(s) may be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing [see, e.g., Caron et al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-2922 (1992). Homodimeric antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci., 94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers and disclose that such homodimers can enhance apoptotic activity as compared to the monomers. Alternatively, the antibodies can be engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer 35 Drug Design, 3:219-230 (1989)].

D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells.

40 The antibody is preferably administered to the mammal in a carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing

5 Co., edited by Oslo *et al.* Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more
10 preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, liposomes or microparticles.
15 It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of antibody being administered.

20 The antibody can be administered to the mammal by injection (*e.g.*, intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The antibody may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred.

25 Effective dosages and schedules for administering the antibody may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of antibody that must be administered will vary depending on, for example, the mammal which will receive the antibody, the route of administration, the particular type of antibody used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody is found in the literature on therapeutic uses of antibodies, *e.g.*, Handbook of Monoclonal Antibodies, Ferrone *et al.*, eds., Noges Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith *et al.*, Antibodies in Human Diagnosis and Therapy, Haber *et al.*, eds., Raven Press, New York 35 (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 μ g/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

40 The antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic

5 agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and cytokines. The antibody may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of antibody and therapeutic agent depend, for example, on what
10 type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually.

Following administration of antibody to the mammal, the mammal's cancer and physiological condition can be monitored in
15 various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques.

20 The Apo-2 receptor antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2 receptor(s), for instance, through complement fixation or ADCC. Alternatively, antagonistic anti-Apo-2 receptor antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting
25 from NF- κ B activation. Such therapeutic antibodies can be utilized according to the methods and techniques described above.

20 Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or
35 indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as 3 H, 14 C, 32 P, 35 S, or 125 I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the
40 art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature,

5 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

10 Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the 15 material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

E. Kits Containing Apo-2 or Apo-2 Antibodies

20 In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds 25 a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described 30 above.

35 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

40 The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present

5 specification are hereby incorporated by reference in their entirety.

EXAMPLES

10 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

15

EXAMPLE 1

Isolation of cDNA clones Encoding Human Apo-2

20 Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., *Curr. Biol.*, 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows. Reagents were added together and 25 incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xhol, Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol 30 (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

35 The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturer's recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The 40 transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the

5 colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of 10 cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase 15 removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in distilled 20 water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried 30 in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 35 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and 40 centrifuged again for 2 minutes at 14,000 x g. The supernatant was

5 removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3 ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1
10 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

15 The cDNA libraries were screened by hybridization with a synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGG
(SEQ ID NO:3) based on the EST.

20 Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

25 The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., *supra*] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted 30 polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular 35 domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53. Apo-2 polypeptide is obtained or obtainable by expressing the molecule encoded by the cDNA 40 insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by

5 the presence of multiple (usually four) cysteine-rich domains in their
extracellular regions -- each cysteine-rich domain being approximately
45 amino acids long and containing approximately 6, regularly spaced,
cysteine residues. Based on the crystal structure of the type 1 TNF
receptor, the cysteines in each domain typically form three disulfide
10 bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are
paired together. Like DR4, Apo-2 contains two extracellular cysteine-
rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR
family members contain three or more such domains [Smith et al., Cell,
15 76:959 (1994)].

15 The cytoplasmic region of Apo-2 contains a death domain
(amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which
shows significantly more amino acid sequence identity to the death
domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%);
20 or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids
that are required for signaling by TNFR1 [Tartaglia et al., supra] are
conserved in Apo-2 while the other two residues are semi-conserved (see
Fig. 2B).

25 Based on an alignment analysis (using the ALIGN™ computer
program) of the full-length sequence, Apo-2 shows more sequence identity
to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1
(19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP)
(29%).

EXAMPLE 2

A. Expression of Apo-2 ECD

30 A soluble extracellular domain (ECD) fusion construct was
prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1)
was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma).
35 (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1
to provide flexibility at the junction, even though residues 183 and 184
are predicted to be in the transmembrane region). The Flag epitope-
tagged molecule was then inserted into pRK5, and expressed by transient
transfection into human 293 cells (ATCC CRL 1573).

40 After a 48 hour incubation, the cell supernatants were
collected and either used directly for co-precipitation studies (see
Example 3) or subjected to purification of the Apo-2 ECD-Flag by
affinity chromatography on anti-Flag agarose beads, according to

5 manufacturer's instructions (Sigma).

B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge 10 and Fc region of human immunoglobulin G₁ heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., 15 supra.

EXAMPLE 3

Immunoprecipitation Assay Showing Binding Interaction

Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate 20 with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) 25 (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl 30 anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By 35 using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then 40 detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying 40 Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™

5 instrument. The BIACORE™ analysis indicated a dissociation constant (K_d) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., *Nature*, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis 10 Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

EXAMPLE 4

Induction of Apoptosis by Apo-2

15 Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., *supra*, Nagata et al., *supra*]. To determine whether Apo-2 was capable of 20 inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount 25 of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., *Curr. Biol.*, 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

30 For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means \pm SEM of at least three experiments, as described in Marsters et al., *Curr. Biol.*, 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research 35 Biochemicals Intl.) were added at 200 μ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

40 FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., *supra*], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et

5 al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant
form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-
3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al.,
supra] did not inhibit apoptosis induction by Apo-2 when co-transfected
10 into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2
signals apoptosis independently of FADD. Consistent with this
conclusion, a glutathione-S-transferase fusion protein containing the
Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and
translated FADD (data not shown).

15

EXAMPLE 5

Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

20 Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et
al., supra) was pre-incubated for 1 hour at room temperature with PBS
buffer or affinity-purified Apo-2 ECD (5 µg/ml) together with anti-Flag
25 antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour
incubation, the cells were analyzed for apoptosis by FACS (as above)
(Fig. 4D).

30 Apo-2L induced marked apoptosis in HeLa cells, and the
soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),
confirming a specific interaction between Apo-2L and Apo-2. Similar
results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-
response analysis showed half-maximal inhibition at approximately 0.3 nM
Apo-2 immunoadhesin (Fig. 4E).

35

EXAMPLE 6

Activation of NF-KB by Apo-2

An assay was conducted to determine whether Apo-2 activates
40 NF-KB.

HeLa cells were transfected with pRK5 expression plasmids
35 encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested
24 hours after transfection. Nuclear extracts were prepared and 1 µg of
nuclear protein was reacted with a ³²P-labelled NF-KB-specific synthetic
oligonucleotide probe

40 ATCAGGGACTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al.,
J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold

5 excess of unlabelled probe, or with an irrelevant 32 P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-KB (1 μ g/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an 10 electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-KB activation as measured by the electrophoretic mobility shift assay; 15 the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF-KB inhibited the mobility of the NF-KB probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF-KB activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, 20 soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., *Nature*, 312:721 (1984)) (1 μ g/ml) and assayed for NF-KB activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF-KB activation in the treated HeLa cells but not 25 in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF-KB activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF-KB inhibitor, ALLN (N-acetyl-Leu-Leu-30 norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 μ g/ml) or cyclohexamide (Sigma) (50 μ g/ml) for 1 hour before addition of Apo-2L (1 μ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

35 The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-KB-dependent genes. The data also indicates that Apo-2L is capable of

5 activating NF-KB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

EXAMPLE 7

Northern Blot Analysis

10 Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase ³²P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated 15 with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute 20 wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after 25 overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been 30 shown previously to express DR4 [Pan et al., supral], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

35 Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., 40 Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to

5 the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

10

EXAMPLE 9

Preparation of Monoclonal Antibodies Specific for Apo-2

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5 μ g/50 μ l of an Apo-2 ECD immunoadhesin protein 15 (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G₁ heavy 20 chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified 25 from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra (See also Example 2B above).

25 Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with 30 murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin protein.

35 In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 μ l of 2 μ g/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three 40 times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 200 μ l of 2.0% bovine serum albumin in PBS and incubated at room temperature for 1 hour. The plates

5 were then washed again three times with wash buffer.

After the washing step, 50 μ l of 0.4 μ g/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

10 Following the wash steps, 100 μ l of the hybridoma supernatants or various concentrations of purified antibody (using Protein A-sepharose columns) was added to designated wells in the presence of CD4-IgG. 100 μ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were 15 incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

20 Next, 50 μ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50 μ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped 25 by adding 50 μ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

30 The supernatants testing positive in the ELISA (calculated as approximately 4 times above background) were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25 μ l of cells suspended (at 4×10^6 cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02% NaN_3) were added to U-bottom microtiter wells, mixed with 100 μ l of culture supernatant or purified 35 antibody (purified on Protein A-sepharose columns) (10 μ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100 μ l FITC-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150 μ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson,

5 Mountain View, CA).

Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

10

EXAMPLE 10

Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells (5×10^5 cells/0.1ml) were incubated with varying concentrations of antibodies in 100 μ l complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C with or without 10 μ g of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300 μ l of complete RPMI. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO₂. The cells were then harvested and washed once with PBS. The apoptosis of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200 μ l binding buffer. Ten μ l of annexin-V-FITC (1 μ g/ml) and 10 μ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

EXAMPLE 11Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells (5×10^5 cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were preincubated with 10 μ g of antibodies in 200 μ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5 μ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., supra) was suspended into complete RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO₂. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech).

Specifically, the cells were washed in PBS and resuspended in 200 μ l binding buffer. Ten μ l of annexin-V-FITC (1 μ g/ml) and 10 μ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody.

EXAMPLE 12ELISA Assay to Test Binding of Apo-2 Antibodies to Other Apo-2 Ligand Receptors

An ELISA was conducted to determine if the monoclonal antibody described in Example 9 was able to bind other known Apo-2L receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 9 above.

The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.

EXAMPLE 13Antibody Isotyping

The isotype of the 3F11.39.7 antibody (as described above) was determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 9 above). The wells in the microtiter plates were then blocked with 200 µl of 2% bovine serum albumin and incubated at room temperature for one hour. The plates were washed again three times with wash buffer. Next, 100 µl of 5 µg/ml of purified 3F11.39.7 antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 µl HRP-conjugated goat anti-mouse IgG (as described above) was added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 3F11.39.7 antibody is an IgG1 antibody.

EXAMPLE 14Preparation of Monoclonal Antibodies Specific for DR4

Further monoclonal antibodies to Apo-2 were produced essentially as described in Example 9 above. Using the capture ELISA described in Example 9, additional anti-Apo-2 antibodies, referred to as 2B3.7.1; 4B9.23.6; 5C7.9.1; 3C9.8.6; and 4H10.14.10, were identified (see Table

5 in Figure 12). Analysis by FACS (using the technique described in
Example 9) confirmed binding of these antibodies to 9D cells
expressing Apo-2 (data not shown).

Three additional anti-Apo-2 antibodies, referred to as 3H1.18.10,
3H3.14.5 and 3D5.1.10, were produced using a mixed antigen
10 immunization protocol as follows. Animals were immunized with four
receptor immunoadhesins - DR4 [Pan *et al.*, Science, 276:111-113
(1997)]; Apo-2 [as described herein]; DcR1 [Sheridan *et al.*, Science
277:818-821 (1997)]; and DcR2 [Marsters *et al.*, Curr. Biol., 7:1003-1006
(1997)]. Receptor immunoadhesins (designated "DR4-IgG", "Apo-2-IgG",
15 "DcR1-IgG" and "DcR2-IgG") were prepared by fusing the extracellular
domain sequence of each receptor to the hinge and Fc region of human
immunoglobulin G₁ heavy chain in pRK5 as described previously [Ashkenazi
et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The
immunoadhesin proteins were expressed by transient transfection into
20 human 293 cells and purified from cell supernatants by protein A
affinity chromatography, as described by Ashkenazi *et al.*, *supra*.
Purified immunoadhesin was suspended in phosphate buffered saline (PBS).
25

Balb/C mice (from Charles River Laboratories) were immunized into
each hind foot pad 14 times at 3-4 day intervals, with a mixture of
DR4-IgG, Apo-2-IgG, DcR1-IgG and DcR2-IgG (1 μ g each) suspended in
30 monophosphoryl lipid A plus trehalose dicorynomycolate adjuvant (MPL-
TDM; Ribi Immunochem. Research Inc., Hamilton, MT) at a 1:1 ratio of
immunoadhesin:adjuvant (DcR2-IgG was only included in the mixture
used for the final six immunizations).

Three days after the final boost, popliteal lymph node cells
35 nodes were removed from the mice and a single cell suspension was
prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented
with 1% penicillin-streptomycin. The lymph node cells were fused with
murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene
glycol and cultured in 96-well culture plates.

Hybridomas were selected in super DMEM [DMEM plus 10% fetal calf
serum (FCS), 10% NCTC-109 (BioWittaker, Wakersville, MD), 100 mM
pyruvate, 100 U/ml insulin, 100 mM oxaloacetic acid, 2 mM glutamine,
40 1% nonessential amino acids (GIBCO), 100 U/ml penicillin and 100 μ g/ml
streptomycin] containing 100 μ M hypoxanthine, 0.4 μ M aminopterin, and

5 16 μ M thymidine (1x HAT, Sigma Chemical Co., St. Louis, MO).

Ten days after the fusion, 180 μ l of each hybridoma culture supernatant was screened for the presence of antibodies to three different antigens (i.e. DR4-IgG, Apo-2-IgG and CD4-IgG control) in a capture ELISA. Hybridoma cells were re-fed with 200 μ l of super DMEM 10 containing 10% FCS and antibiotics. Two days later, 180 μ l of culture supernatant was collected and screened for the presence of antibodies to another two different antigens (i.e. DcR1-IgG and DcR2-IgG) in a capture ELISA. After careful examination of the ELISA results, potential positive hybridomas secreting monoclonal antibodies against 15 each antigen were cloned twice using a limiting dilution method.

EXAMPLE 15

Antibody Isotyping

The isotypes of the 2B3.7.1; 4B9.23.6; 5C7.9.1; 3H1.18.10; 20 3C9.8.6; 4H10.14.10; 3H3.14.5 and 3D5.1.10 anti-Apo-2 antibodies (described in Example 14) were determined essentially as described in Example 13.

The isotyping analysis showed that the 2B3.7.1; 5C7.9.1; 3H1.18.10; 3H3.14.5 and 3D5.1.10 antibodies are IgG1 antibodies. Anti-Apo-2 antibodies 4B9.23.6 and 4H10.14.10 are IgG2a antibodies, 25 and antibody 3C9.8.6 is an IgG2b antibody. (See, also, Figure 12)

EXAMPLE 16

Determination of Monoclonal Antibody Affinities

The equilibrium dissociation and association constant rates of 30 two of the Apo-2 antibodies (described in the Examples above) were determined using KinExA™, an automated immunoassay system (Sapidyne Instruments, Inc., Boise, ID), as described with a modification by Blake et al., Journal of Biological Chemistry, 271:27677-685 (1996); 35 and Craig et al., Journal of Molecular Biology, 281:183-201 (1998). Briefly, 1.0 ml of anti-human IgG agarose beads (56 μ m, Sigma, St. Louis, MO) were coated with 20 μ g of Apo-2-IgG (described in Example 9) in PBS by gentle mixing at room temperature for 1 hour. After washing with PBS, non-specific binding sites were blocked by 40 incubating with 10% human serum in PBS for 1 hour at room temperature.

5 A bead pack (~4 mm high) was created in the observation flow cell
by the KinExA™ instrument. The blocked beads were diluted into 30 ml
of assay buffer (0.01% BSA/PBS). The diluted beads (550 μ l) were next
drawn through the flow cell with a 20 μ m screen and washed with 1 ml
of running buffer (0.01% BSA; 0.05% Tween 20 in PBS). The beads were
10 then disrupted gently with a brief backflush of running buffer,
followed by a 20 second setting period to create a uniform and
reproducible bead pack. For equilibrium measurements, the selected
Apo-2 antibodies (3F11.39.7 and 3H3.14.5) (5 ng/ml in 0.01 % BSA/PBS)
were mixed with a serial dilution of Apo-2-IgG (starting from 2.5 nM
15 to 5.0 pM) and were incubated at room temperature for 2 hours. Once
equilibrium was reached, 4.5 ml of this mixture was drawn through the
beads, followed by 250 μ l of running buffer to wash out the unbound
antibodies. The primary antibodies bound to beads were detected by
1.5 ml of phycoerythrin labeled goat anti-mouse IgG (Jackson
20 Immunoresearch). Unbound labeled material was removed by drawing 4.5
ml of 0.5 M NaCl through the bead pack over a 3 minute period. The
equilibrium constant was calculated using the software provided by the
manufacturer (Sapidyne, Inc.).

25 The affinity determinations for the Apo-2 antibodies are shown in
Figure 13. Affinity determinations for immunoadhesin constructs of
the DR4 and Apo-2 receptors for Apo-2L are shown for comparison. The
affinities (Kd-1) of the antibodies 3F11.39.7 and 3H3.14.5 were 20 pM
and 5 pM, respectively, demonstrating that both antibodies have high
affinities.

30

EXAMPLE 17

Apoptosis Assay of Human Tumor Cell Lines

35 The apoptotic activities of the Apo-2 monoclonal antibodies were
further examined in assays to determine the cell viability of cancer
cells after treatment with the antibodies or Apo-2L.

SKMES-1 cells (human lung tumor cell line; ATCC), Colo205 cells
(human colon tumor cell line; ATCC), or G142 human glioma cells (Dr.
Manfred Westphal, Universitats Krankenhaus Eppendorf, Hamburg,
Germany) were seeded at 4×10^4 cells/well in complete high glucose 50:50
40 medium supplemented with glutamine, penicillin and streptomycin, in
tissue culture plates and allowed to attach overnight at 37°C. The

5 media was then removed from the wells, and 0.1 ml of antibody (anti-Apo-2 antibodies diluted 0.001-10 microgram/ml in complete medium) was added to selected wells. Control wells without antibody received a media change with or without Apo-2L. The plates were then incubated for 1 hour at room temperature.

10 The culture supernatant was removed from the wells containing the test antibodies, and 10 microgram/ml goat anti-mouse IgG-Fc (Cappel Laboratories) was added to the wells. Media was changed in the control wells. The plates were incubated overnight at 37°C. As a control, Apo-2L (as described in Example 11) (in potassium phosphate buffer, pH 7.0) was diluted to 2 microgram/ml. 0.1 ml of the diluted Apo-2L solution was added to selected wells, and then serial three-fold dilutions were carried down the plate.

15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 Culture supernatants were then removed from the wells by aspiration, and the plates were flooded with 0.5% crystal violet in methanol solution. After 15 minutes, the crystal violet solution was removed by flooding the plates with running tap water. The plates were then allowed to dry overnight.

Absorbance was read on an SLT 340 ATC plate reader (Salzburg, Austria) at 540 nm. The data was analyzed using an Excel macro and 4p-fit. The results illustrating the activity of the Apo-2 antibodies on SKMES cells are shown in Figure 14. Figure 14 shows that all the monoclonal antibodies tested (except 3H1.18.10) induced apoptosis when the monoclonal antibodies were linked by the addition of goat anti-mouse IgG-Fc antibodies. The most potent apoptotic activity was observed with antibody 3H3.14.5. The EC50 of the 3H3.14.5 antibody was approximately 10 ng/ml, while the EC50 of Apo-2 ligand was approximately 100 ng/ml, suggesting that the 3H3.14.5 antibody may have more potent apoptotic activity than Apo-2 ligand. In the absence of the goat anti-mouse IgG-Fc linker, the monoclonal antibodies did not demonstrate significant apoptotic activity. This result suggests it may be important for oligomerization of Apo-2 receptors to occur in order to induce apoptosis.

The results illustrated in Figure 15A and 15B show the activity of the Apo-2 antibodies on the Colo205 colon cancer cells and glioma cells, respectively. As shown above for the SKMES cells, the antibodies 3F11.39.7 and 3H3.14.5 demonstrated potent apoptotic activities on the Colo205 cells. The 3H3.14.5 antibody appeared to be

5 more potent than Apo-2 ligand. Even more striking effects of the
3H3.14.5 and 3F11.39.7 antibodies were obtained with the glioma G142
cells (which express Apo-2 receptor but not DR4 receptor). At 1
microgram/ml, Apo-2 ligand induced approximately 50% killing of the
G142 cells. In contrast, the 3H3.14.5 and 3F11.39.7 antibodies (at
10 0.1 microgram/ml) could induce approximately 90% killing of the G142
tumor cells. These results suggest that in cancer cells expressing
Apo-2 receptor but not DR4, such anti-Apo-2 antibodies may be more
potent apoptotic inducing agents.

15 EXAMPLE 18

ELISA Assay to Test Binding of Apo-2 Antibodies to Other Apo-2L
Receptors

20 An ELISA assay was conducted (as described in Examples 9 and 12)
to determine binding of the Apo-2 antibodies to other known Apo-2L
receptors, beside Apo-2.

25 The results are shown in Figure 12 and 16. Antibody 3D5.1.10
demonstrated a significant level of binding to DR4 although it did not
bind to DcR1 and DcR2. Antibodies 3F11.39.7 and 3H1.18.10 bind Apo-2
specifically while antibody 3H3.14.5 demonstrated a low level of cross
reactivities to DR4 and DcR1.

EXAMPLE 19

Determination of Blocking Activity of anti-Apo-2 Antibodies

30 In a capture ELISA, 96-well microtiter plates (Maxisorb;
Nunc, Kamstrup, Denmark) were coated by adding 50 μ l of 2 μ g/ml goat
anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each
well and incubating at 4°C overnight. The plates were then washed three
times with wash buffer (PBS containing 0.05% Tween 20). The wells in
35 the microtiter plates were then blocked with 200 μ l of 2.0% bovine serum
albumin in PBS and incubated at room temperature for 1 hour. The plates
were then washed again three times with wash buffer.

40 After the washing step, 100 μ l of various concentrations of
anti-Apo-2 antibodies in assay buffer was added to each well. The
plates were incubated for 1 hour at room temperature on a shaker
apparatus, followed by washing three times with wash buffer.

Following the wash steps, the plate wells were incubated with

5 50 μ l/well of 10 ng/ml of Apo-2L (amino acids 114-281; prepared as
described in Example 11). The plates were incubated at room temperature
for 1 hour on a shaker apparatus. The Apo-2L bound to the wells was
then detected by the addition of biotinylated anti-Apo-2L monoclonal
antibody, 2E11 (ATCC HB-12256), followed by the addition of
10 streptavidin.

15 The results are illustrated in Figure 17. Antibodies 3H3.14.5,
3F11.39.7, 3C9.8.6, and 4H10.14.10 inhibited the binding of Apo-2
ligand to the Apo-2 receptor captured to the goat anti human IgG-
coated ELISA wells, demonstrating that these antibodies are blocking
antibodies. Antibody 3H3.14.5 appeared to have the strongest blocking
activity. The blocking activities of the antibodies are also
summarized in Figure 12.

EXAMPLE 20

In vivo Activity of Apo-2 Antibodies

20 Colo205 cells (human colon tumor cell line; ATCC) were grown in
high glucose F-12:DMEM (50:50) medium supplemented with 10% FCS, 2 mM
glutamine, 100 μ g/ml of penicillin, and 100 μ g/ml streptomycin. The
cells were harvested after treating with cell dissociation medium
25 (Sigma, IAC) for 5 minutes. After washing in PBS, the tumor cells
were resuspended in PBS at a concentration of 3×10^7 cells/ml.

30 Nude mice were injected with $3-5 \times 10^6$ cells subcutaneously in
the dorsal area in a volume of 0.1 ml. Control animals received
either a control IgG1 antibody or PBS vehicle alone. When the tumor
size in the Colo205 tumor bearing animals became a desired size, the
mice were injected i.p. with 100 μ g of 3F11.39.7 or 3H3.14.5 anti-Apo-
2 antibody in PBS three times per week, and the tumor sizes were
measured three times/week.

35 The results illustrated in Figure 18 show that both 3F11.39.7
antibody and 3H3.14.5 antibody inhibited growth of Colo205 tumors.
Compared to the tumor growth in the group of mice treated with control
IgG1 or none, the group of mice treated with antibody 3H3.14.5 or
3F11.39.7 showed almost no detectable tumor growth. On Day 20, four
40 out of eight mice showed no visible tumor growth after treatment with
antibody 3H3.14.5, while two out of eight mice showed no visible tumor
growth after treatment with antibody 3F11.39.7. The results suggest

5 that the monoclonal antibodies alone may be potent anti-cancer therapeutic agents. It is presently believed that component(s) in the treated animals' serum may link the administered (IgG1) monoclonal antibodies, resulting in the potent agonistic anti-cancer activity observed.

10

EXAMPLE 21

Crystal Structure Analysis of Apo-2 Receptor/Apo-2L Complex

15 Experiments were conducted to determine the crystal structure of the complex between Apo-2L and an extracellular domain sequence of Apo-2. As described below, the structure revealed three thin, elongated receptors snuggled into long crevices between pairs of monomers of the homotrimeric Apo-2 ligand. The interface is divided into two distinct patches, one near the "bottom" of the complex close to the receptor cell surface and one near the "top" closest to the ligand cell surface. Both patches contain residues that are believed to be critical for high-affinity binding. It is believed that the top patch may recognize conserved hydrophobic features, while the bottom patch may control specificity and cross-reactivity.

20 1. Expression and purification

25 Apo-2L (residues 114-281; amino acid sequence provided in Pitti et al., supra) was expressed in *E. coli* and purified as described in Ashkenazi et al., J. Clin. Invest., 104:155-162 (1999). Apo-2 (residues 1-130; see Fig. 1; SEQ ID NO:1) was expressed in Hi5 insect cells (Expression Systems LLC, Woddland, CA) with a baculovirus 30 transfer vector (PharMingen, San Diego, CA) under the control of a polyhedron promoter. Protein was secreted from the cells grown at 27°C over 72 hours. The Apo-2-containing medium was separated from the cells by centrifugation. The supernatant was run over a Q-sepharose (Pharmacia) column and the protein eluted with a 0-1M NaCl 35 gradient in 20mM Tris-HCl, pH 8.0. The fractions containing Apo-2 were pooled and loaded onto a CNBr-Apo-2L affinity column. The column was washed with 0.5M NaCl in 20mM Tris-HCL, pH 8.0, and Apo-2 was eluted with 2M KSCN in 50mM Tris, pH 8.0. Apo-2 was further purified 40 by size exclusion chromatography (S-200, Pharmacia). Apo-2L in 20mM Tris-HCl, pH 8.0 was added to purified Apo-2 in approximately equimolar concentrations, and the complex was purified by size

5 exclusion chromatography S-75, Pharmacia) in 100 mM NaCl, 20mM Tris-HCl, pH 8.0. The fraction containing the Apo-2L/Apo-2 complex was further purified by anion exchange chromatography (MonoQ, Pharmacia) and eluted with a 0-1M NaCl gradient in 20mM Tris, pH 8.0. The complex was then concentrated to approximately 3.7 mg/mL and buffered
10 with 20mM Tris-HCl, pH 8.0, 0.1 M NaCl.

2. Crystallization and data collection

Crystals of the Apo-2L/Apo-2 complex were grown by vapor diffusion at 19°C using the hanging drop method. The initial crystals were grown in condition 37 of the Hampton Crystal Screen II (10% PEG 15 8000, 8% ethylene glycol, 0.1 M Hepes, pH 7.5). The crystals used for data collection were grown by mixing 2 microliter of protein solution with 2 microliter of reservoir consisting of 15% PEG 8000, 10% ethylene glycol, 0.2M ammonium sulfate, 0.1M Tris-HCl, pH 7.5, and grew to a size of 0.3 mm x 0.15 mm x 0.1 mm. The crystals were transferred briefly to a droplet containing reservoir solution with 20% glycerol before flash-cooling in liquid nitrogen. The crystals belonged to space group $P_{2}12_{1}2_{1}$ and had unit cell dimensions $a=66.8 \text{ \AA}$, $b=112.0 \text{ \AA}$, $c=130.8 \text{ \AA}$. The asymmetric unit contained one Apo-2L trimer and three receptor molecules. A 3.5 Å data set was collected on a MAR imaging plate system using a Rigaku rotating anode generator with CuKalpha radiation. A subsequent data set to 2.4 Å resolution was collected from a single crystal at beam line 7-1 of the Stanford Synchrotron Radiation Laboratory. The data sets were processed using the programs in the HKL package (Otwinowski and Minor, Methods Enzymol., 176:307-326 (1997)).

3. Structure determination and refinement

The Apo-2/Apo-2L structure was determined by molecular replacement using the structure of Apo-2L alone as the search model in combination with three-fold non-crystallographic symmetry (NCS) map 35 averaging. Using all data from 8-4 Å, the program AMoRe (CCP4, Acta Cryst., D50:760-763 (1994)) gave a clear rotation solution with a correlation coefficient of 30.4% (the nearest incorrect peak had a correlation coefficient of 7.3%). The top solution to the translation function had an initial R_{free} (Brünger, Nature, 355:472-475 (1992)) of 40 42.4% following rigid-body fitting using all data between 8 and 3.5 Å. Similar searches using the structure of Apo-2L bound to a homology

5 model of Apo-2 based on the TNF-beta-TNFR1 complex resulted in worse
molecular replacement statistics, but a three-fold NCS-averaged and
solvent-flattened map using program DM (CCP4, supra) revealed partial
density for the receptor. Three cycles of model building, NCS mask
refinement, and density averaging allowed building of receptor
10 residues 22-130. This model was refined with X-PLOR 98.1 (Brünger, X-
PLOR Manual, Version 3.1, New Haven, Connecticut, Yale University
(1992)) as modified by Molecular Simulations, Inc., using a maximum
likelihood target function, NCS constraints, positional refinement,
simulated annealing and grouped B-factors until Rfree reached 33.5%.
15 This partially refined model was then further refined against the 2.4
Å data set, using programs X-PLOR and REFMAC (CCP4, supra).
Examination of sigma weighted 2Fo-Fc and Fo-Fc maps revealed that
differences existed among the three receptor copies, especially
between residues 100 to 130. In subsequent refinement the Apo-2L
20 trimer was subject to tight NCS restraints, while weak restraints were
applied to the most similar regions of the receptors. An overall
anisotropic B-factor correction was applied to the data as was a real
space bulk solvent correction (Brünger, X-PLOR Manual, supra). The
final model consists of three Apo-2L monomers (A, B, and D),
25 containing residues 119-131 and 144-281 (using the amino acid sequence
numbering provided in Pitti et al., supra), and Apo-2 receptor chains
R (residues 21-128) and S and T (residues 22-130). Refinement and
model statistics are shown in Figure 19. The programs Molscript and
Raster3D were used to make figures (Kraulis, J. Appl. Cryst., 24:946-
30 950 (1991); Merrit and Murphy, Acta Cryst., D50:869-873 (1994)).

The complex formed between the extracellular domain sequence of
Apo-2 (residues 1-130) and Apo-2L (residues 114-281) crystallized
readily and was found to contain three receptors and three ligands
assembled as a hexameric complex in the asymmetric unit. Diffraction
35 data were collected using synchrotron radiation, and the structure was
refined to 2.4 Å resolution and an R-value of 23.2% (Rfree of 27.7%;
Figure 19). The final model consists of Apo-2L residues 119-131 and
144-281 in each monomer, and Apo-2 residues 21-128 of one copy and 22-
130 of the other two (Figure 20).

40 Each of the three extensive interaction surfaces buries 2750 Å²
of solvent accessible surface area, 1400 Å² from the receptor and 1350

5 Å² from the ligand. Two receptor loops mediate most of the interactions, dividing the interface in two distinct patches (Figure 21): the "50s loop" (residues 51-65; Fig. 1; SEQ ID NO:1) and the "90s loop" (residues 91-104; Fig. 1, SEQ ID NO:1). In patch A, the 90s loop interacts with a cluster of Apo-2L residues around Gln 205 near 10 the bottom of the trimer, while patch B is formed by the 50s loop of Apo-2 and Apo-2L residues clustered around Tyr 216 near the top of the trimer (Figure 21).

15 Patch A is the larger of the two, with 1790 Å² of total buried accessible surface area (880 Å² from the receptor and 910 Å² from the ligand). The 90s loop of Apo-2 contributes 85% of the buried surface area (750 Å²) while the remaining 130 Å² is a result of small contributions from Apo-2 receptor residues 65-69 and 108, 111, 122, and 125.

20 Patch B is the smaller of the two patches (890 Å² total, 480 Å² from the receptor, 410 Å² from the ligand). Here, the 50s loop of the receptor interacts with the 210s and the 150s loops of Apo-2L. This patch is centered on Apo-2L residue Tyr 216, which binds in a hydrophobic groove on the receptor surface formed by the side chains of Apo-2 residues His 53, Asn 55, Leu 57, Leu 58 and Phe 59. The 25 interactions with the 150s loop are more peripheral and polar in nature, and are primarily mediated through contacts between Arg 62 on the receptor with Apo-2L residues Glu 155 and Ser 159. The actual 30 ligand-binding motif found in CRDs 2 and 3 corresponds to Apo-2 residues 43 to 130.

30 * * * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia, USA (ATCC):

35	<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
	pRK5-Apo-2	209021	May 8, 1997
	3F11.39.7	HB-12456	January 13, 1998
	3H3.14.5	HB-12534	June 2, 1998
	3D5.1.10	HB-12536	June 2, 1998

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules pursuant thereto (including 37 CFR Section 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

5 What is claimed is:

1. A method of inducing apoptosis in mammalian cells expressing Apo-2 receptor comprising exposing mammalian cells expressing Apo-2 receptor to an effective amount of an Apo-2 agonist antibody.
- 10 2. The method of claim 1 wherein said Apo-2 agonist antibody is a monoclonal antibody.
3. The method of claim 1 wherein said Apo-2 agonist antibody is a chimeric antibody.
- 15 4. The method of claim 1 wherein said Apo-2 agonist antibody is a humanized antibody.
5. The method of claim 1 wherein said Apo-2 agonist antibody is a human antibody.
- 20 6. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12456.
- 25 7. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12534.
8. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12536.
- 30 9. The method of claim 2 wherein said monoclonal antibody is produced by the hybridoma deposited as ATCC HB-12535.
- 35 10. A method of treating cancer, comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist antibody.
11. The method of claim 10, wherein said cancer cells are lung cancer cells.
- 40 12. The method of claim 10, wherein said cancer cells are colon cancer cells.

5 13. The method of claim 10, wherein said cancer cells are glioma
cells.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

Fig. 1

Fig. 1 (cont.)

2 A

1 MEORGONAPAASGARAKHGPGRPGLRVPKTLVNVAVLLYBAESALITQOD
61 LAPQORAAPOOKRGSSEGLCPGPGHJISEDGRDCISCKYQDYSSTHMDLFCLRCTBQD
121 SGEVELSPCTTTRNTYCOCECTFREEDSPEMCRKCRTECQPRGMVKVGDCPTWSDIECVH
181 KEGTILIGVTAAVVLLIVAVTUCKSLIMKVLPIYKXGICSGCGDPERVDRSSQBPGED
241 NYMEIRIVSILQPTQVTEQEMEVQEPAAEPTGVMLSPGESEHLLIEPAEAESORRELLIVPA
301 NEGDPTEETLROCFDDFADLVPFTDSWEPLMRKLGLMDNEIKVAKAERAGHDTLTYTMILK
361 VAKTGROASVETLDALETLGERLAKQKIEDHILLSKRMXLEGNADSLSS

Fig. 2 B

apo2
DRA
APO3/DR3
ENFRI
GAS/Apo1

Fig. 3

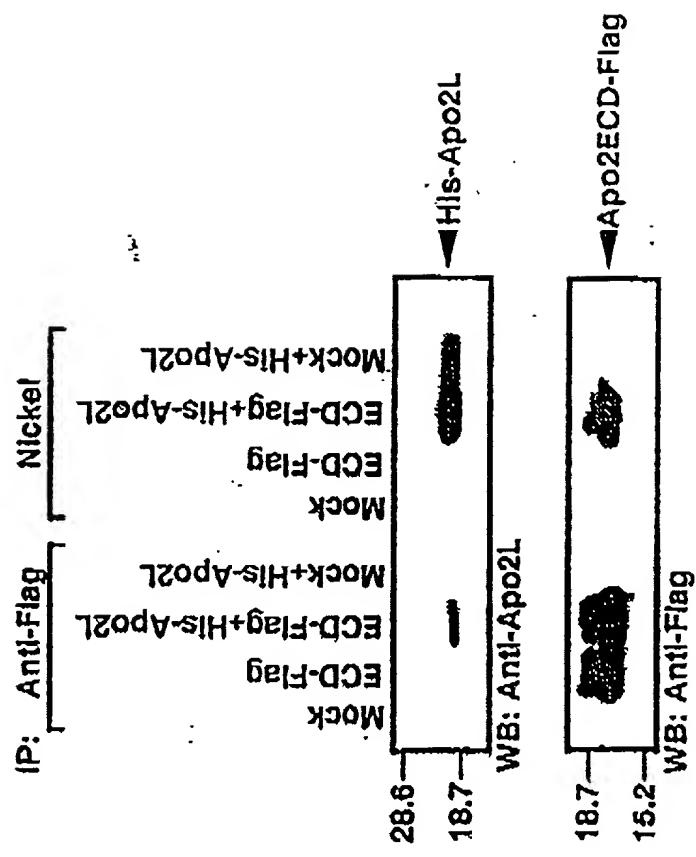
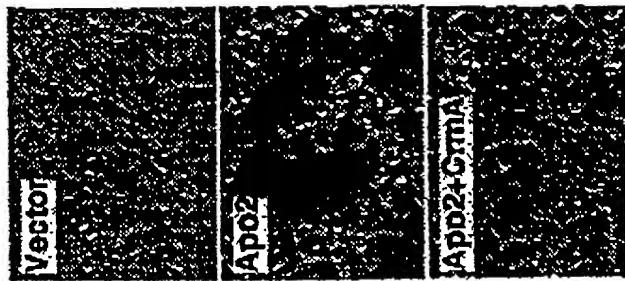
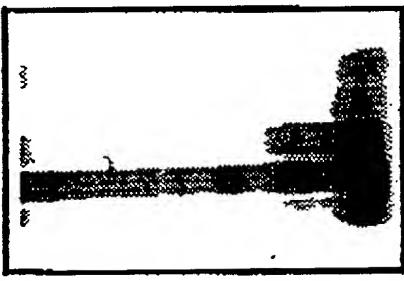


Fig. 4

4 A



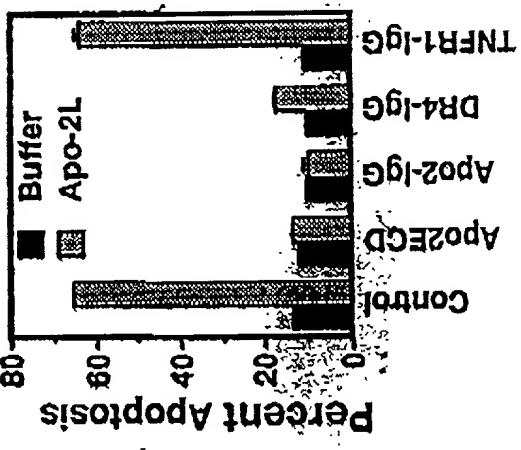
4 B



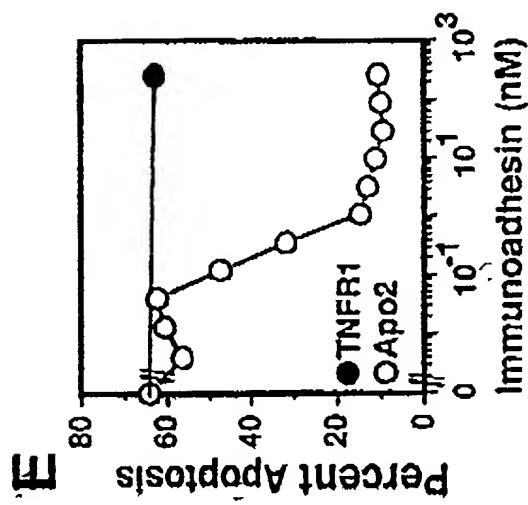
4 C



4 D



4 E



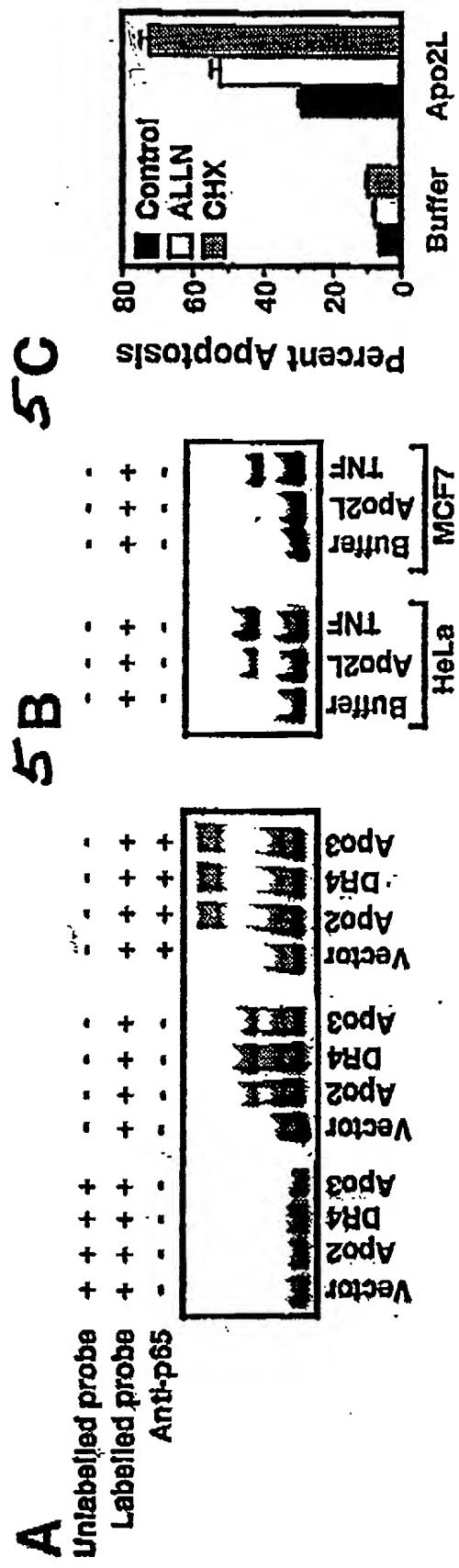
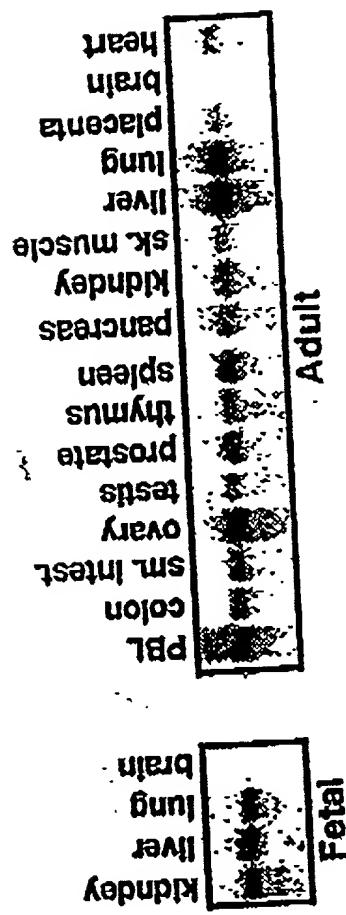


Fig. 5

FIG. 6



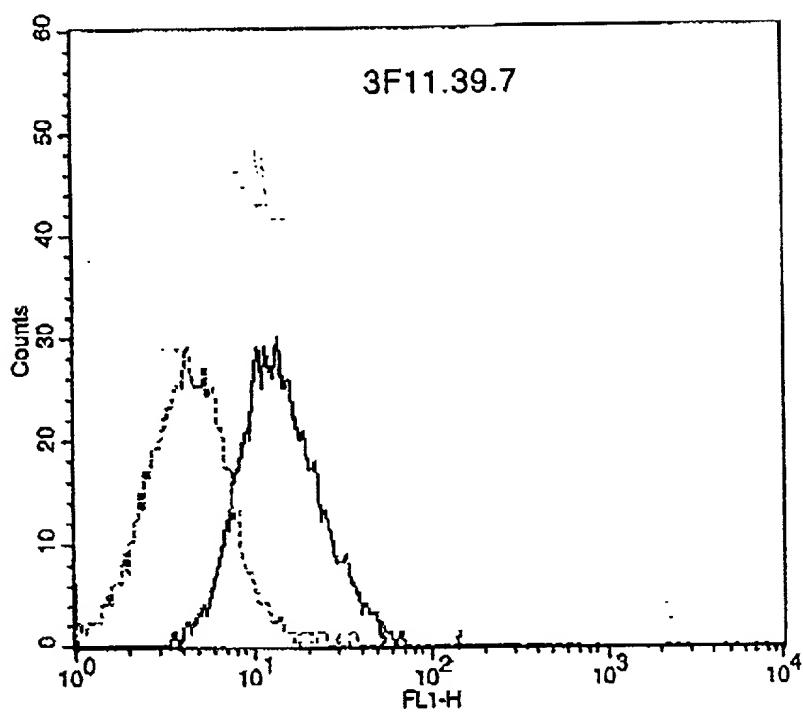


Fig. 7

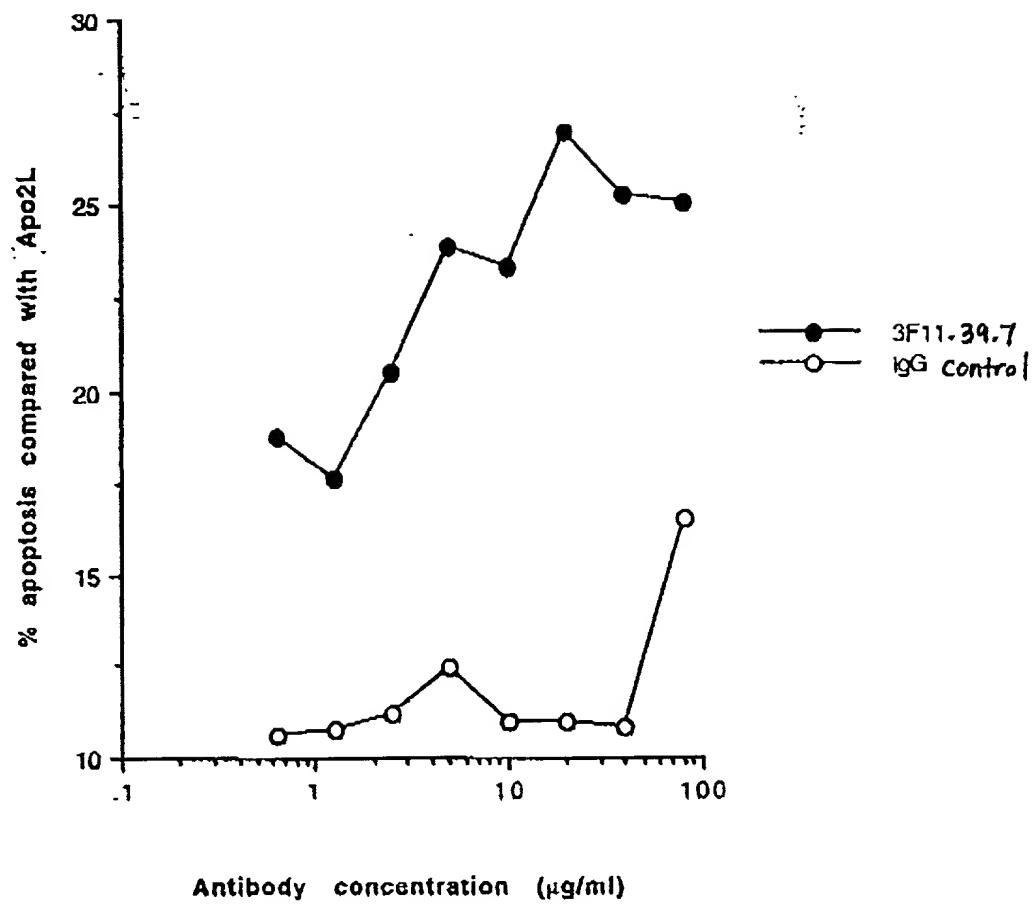


Fig. 8

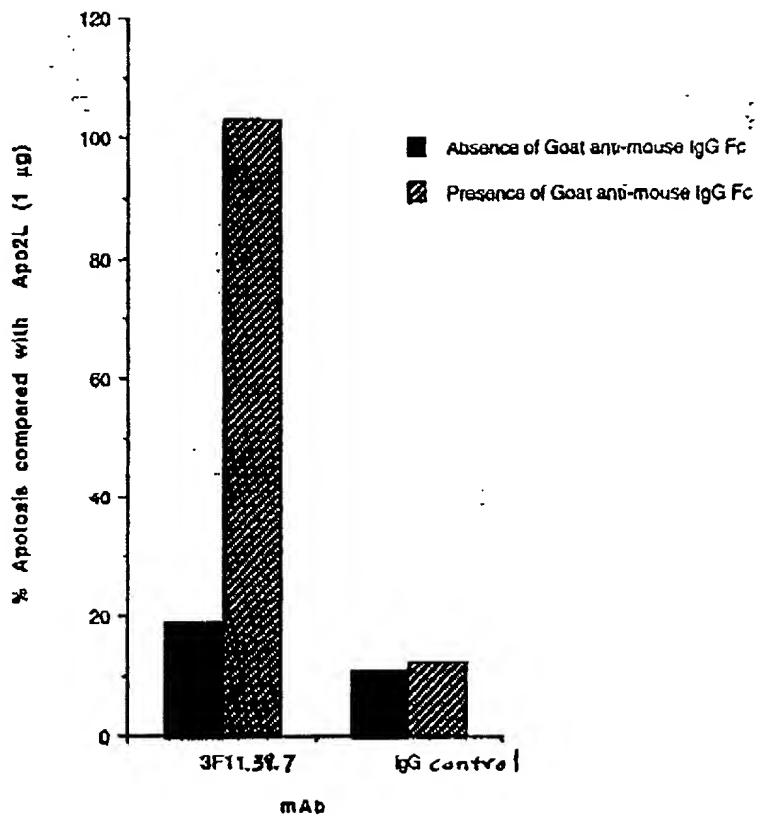


Fig. 9

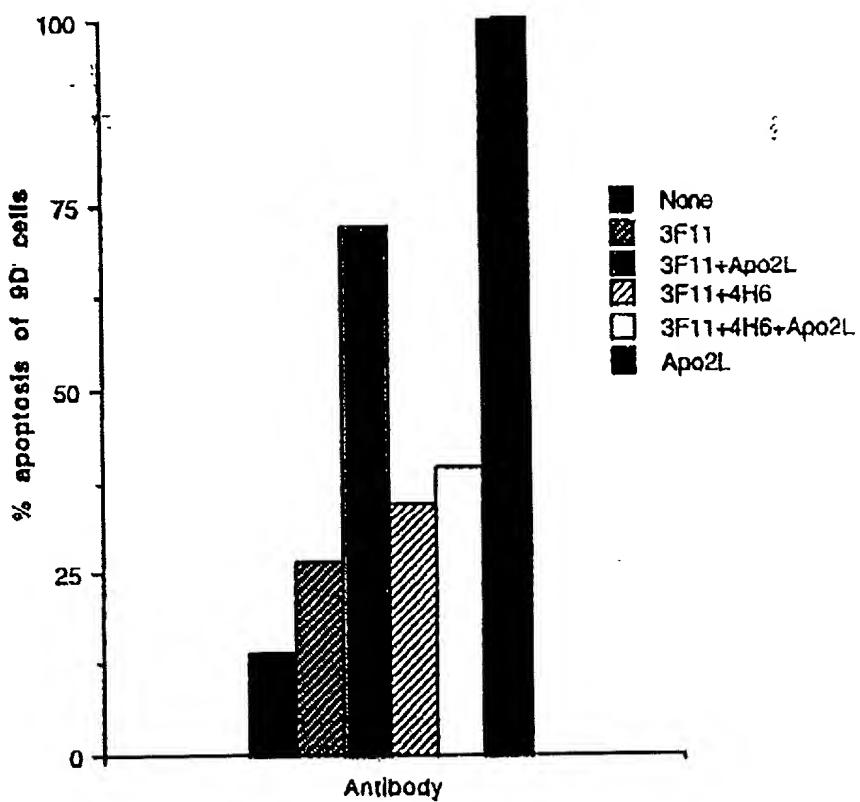


Fig : 10

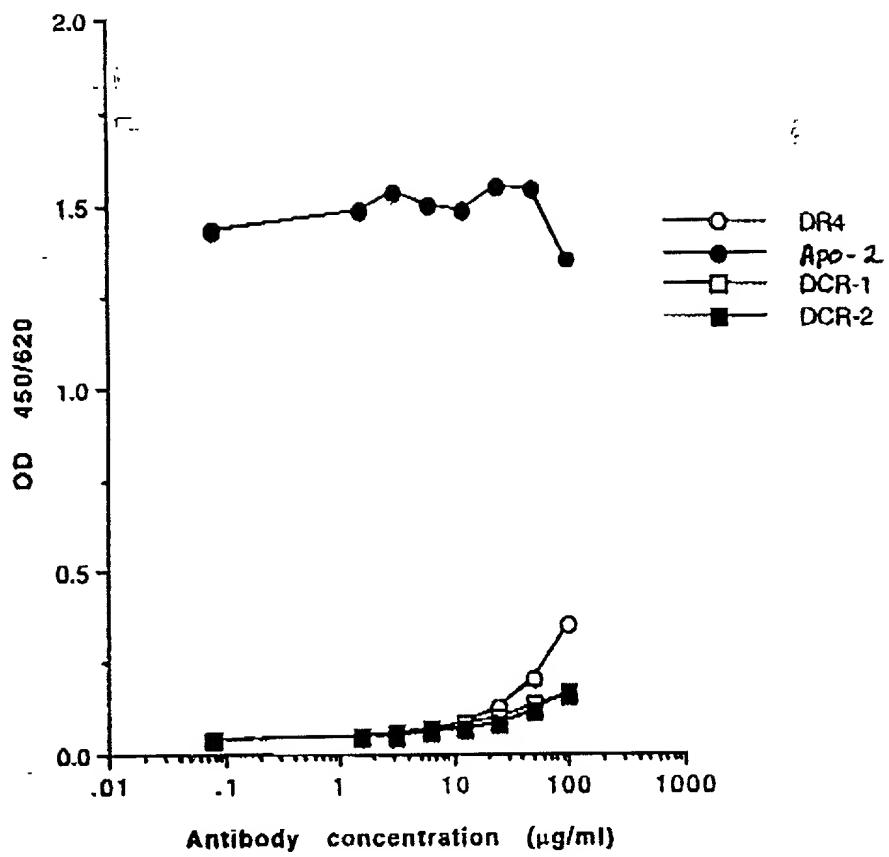


Fig. 11

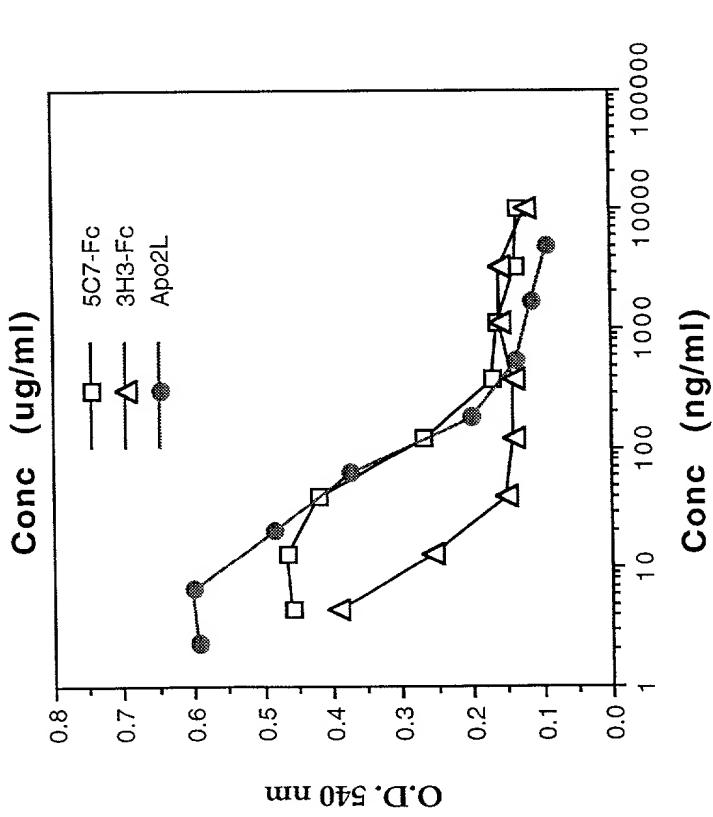
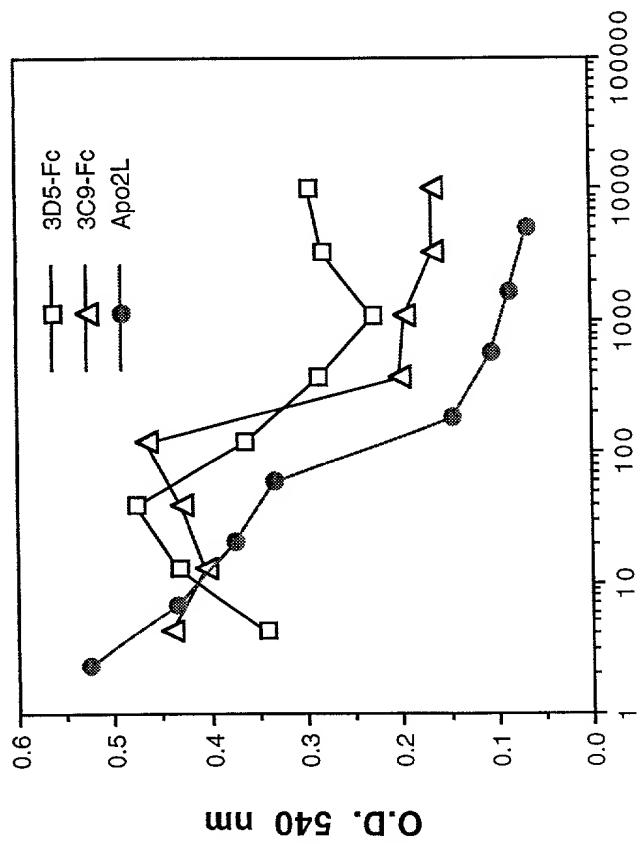
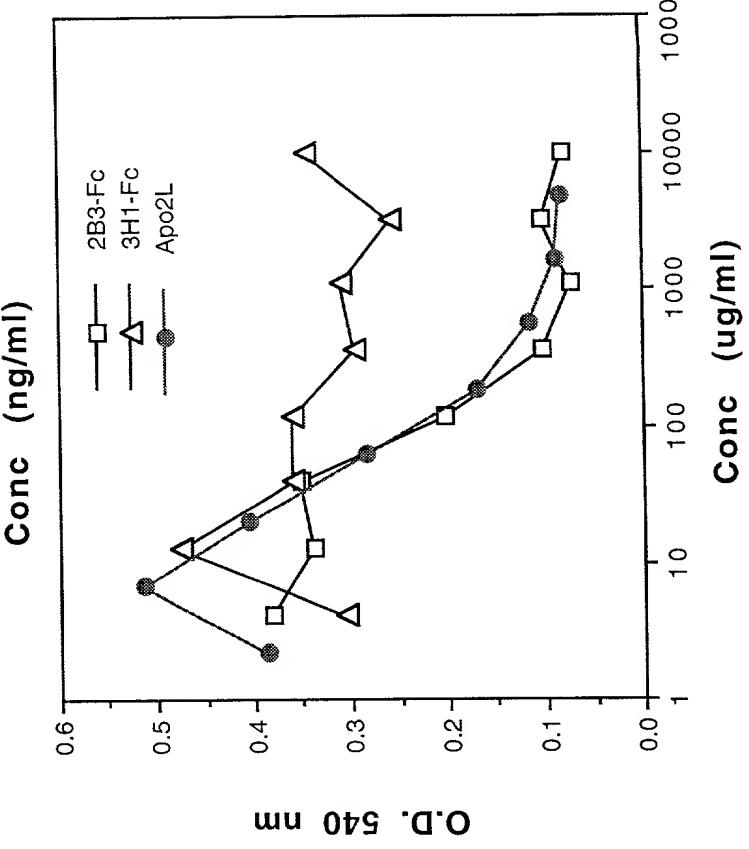
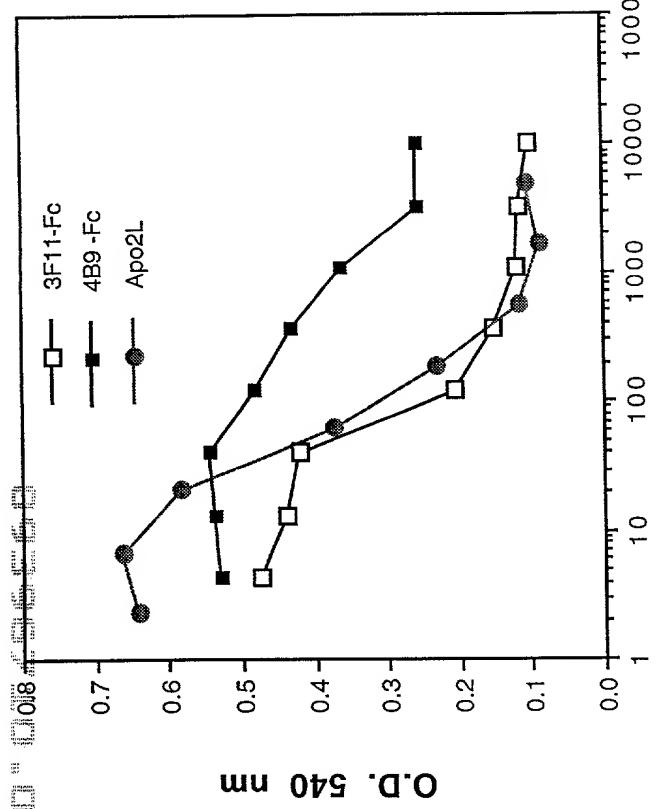
Isotype	Kd-1 (pM)	Bloc	Apop w/L	Cross reactivity			IHC	
				DR4	$A_{\rho\alpha 2}$	DCR1	DCR2	
2B3.7.1	IgG1	-	+	+	++	-	-	-
3F11.39.7	IgG1	20.	+	+	++	-	-	+
4B9.23.6	IgG2a	-	+/-	-	++	-	-	-
5C7.9.1	IgG1	-	+	+	++	-	-	-
3H1.18.10	IgG1	-	-	+	++	-	+/-	+
3H3.14.5	IgG1	4	+	+	++	+	+/-	+
3D5.1.10	IgG1	-	+	++	++	-	-?	-
3C9.8.6	IgG2b	+	?	-	++	-	-	-
4H10.14.10	IgG2a	+/-	+/-	++	++	+/-	+/-	+/-

Fig. 12

		Affinity (pM)
DR4-IgG	to Apo2L	82
<i>Apo-2</i> -IgG	to Apo2L	1
mAb 3F11	to <i>Apo-2</i> IgG	20
mAb 3H3	to "	3

Fig. 13

Fig. 14



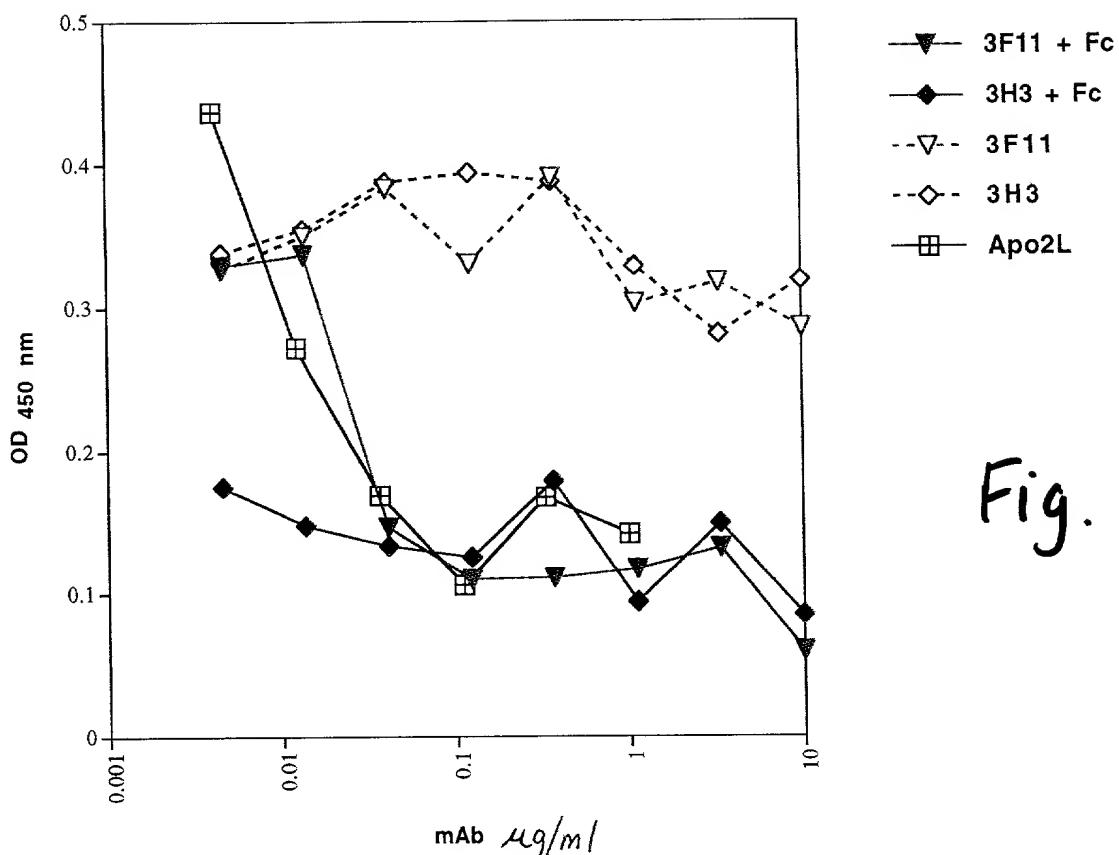


Fig. 15A

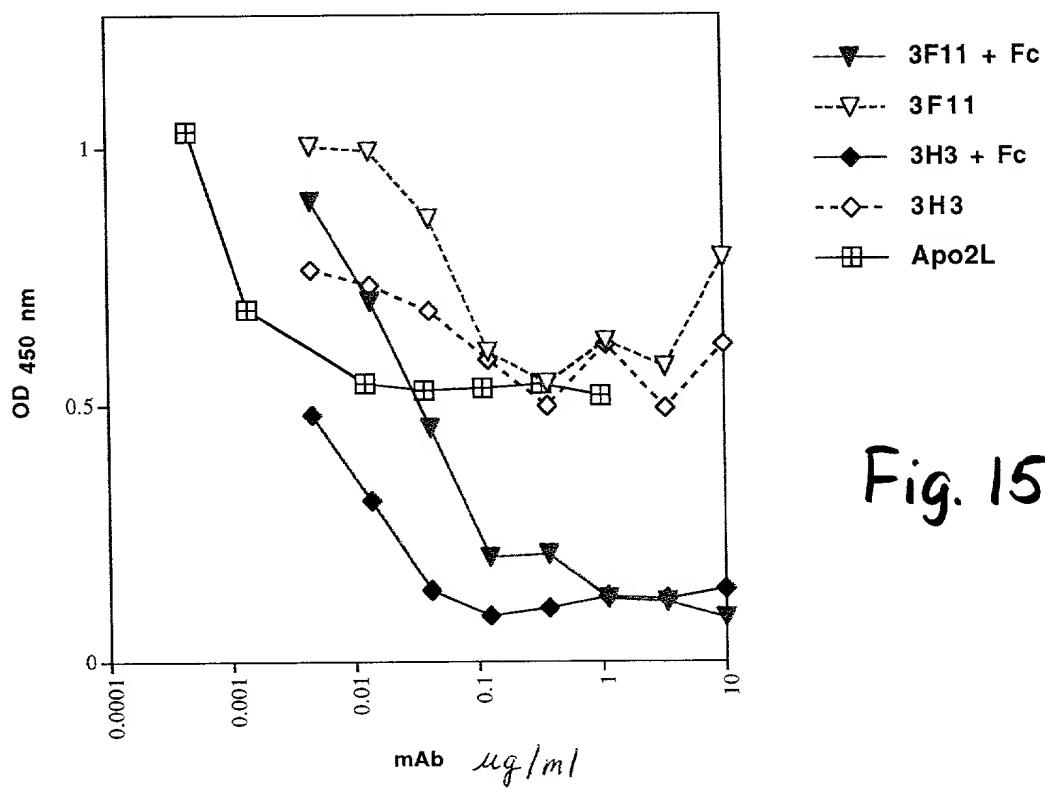


Fig. 15B

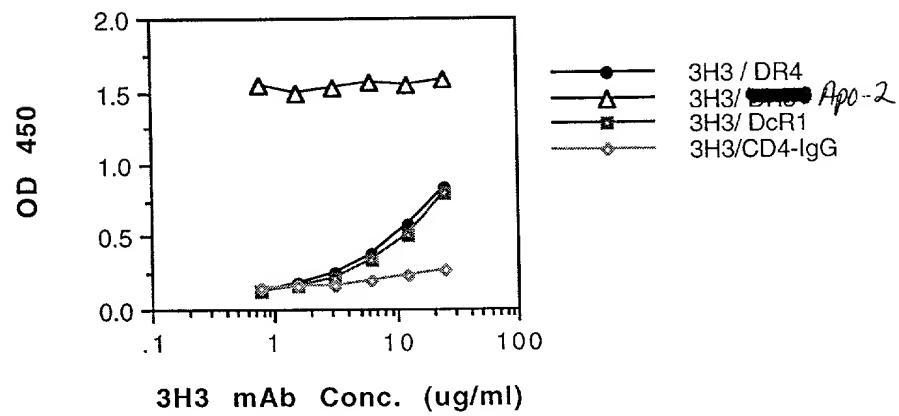
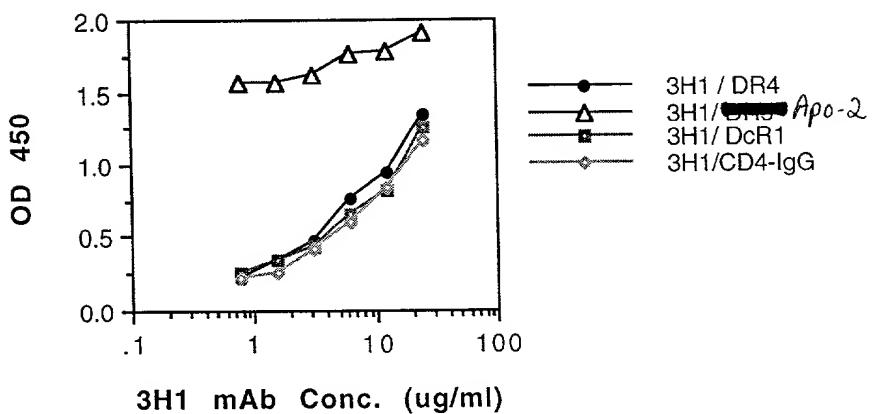
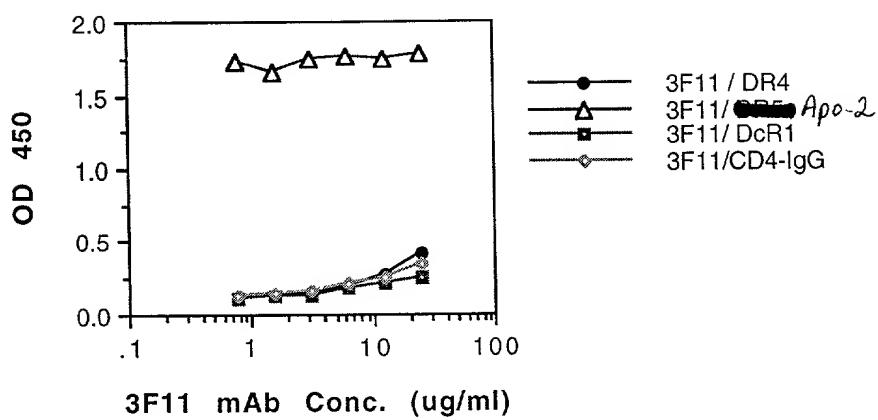
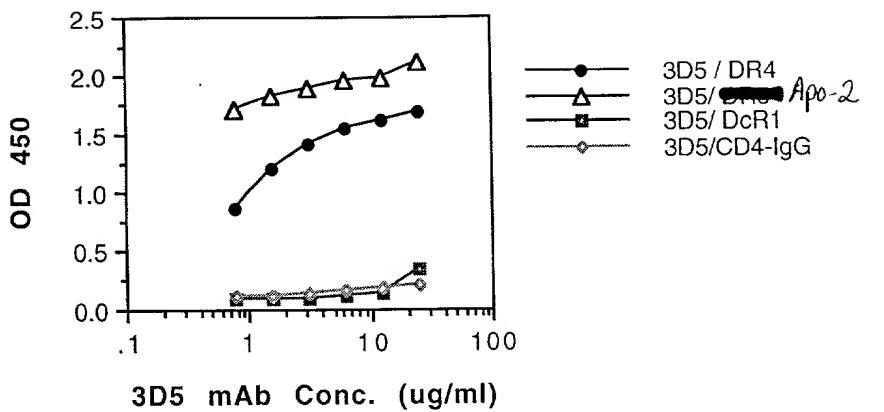


Fig. 16

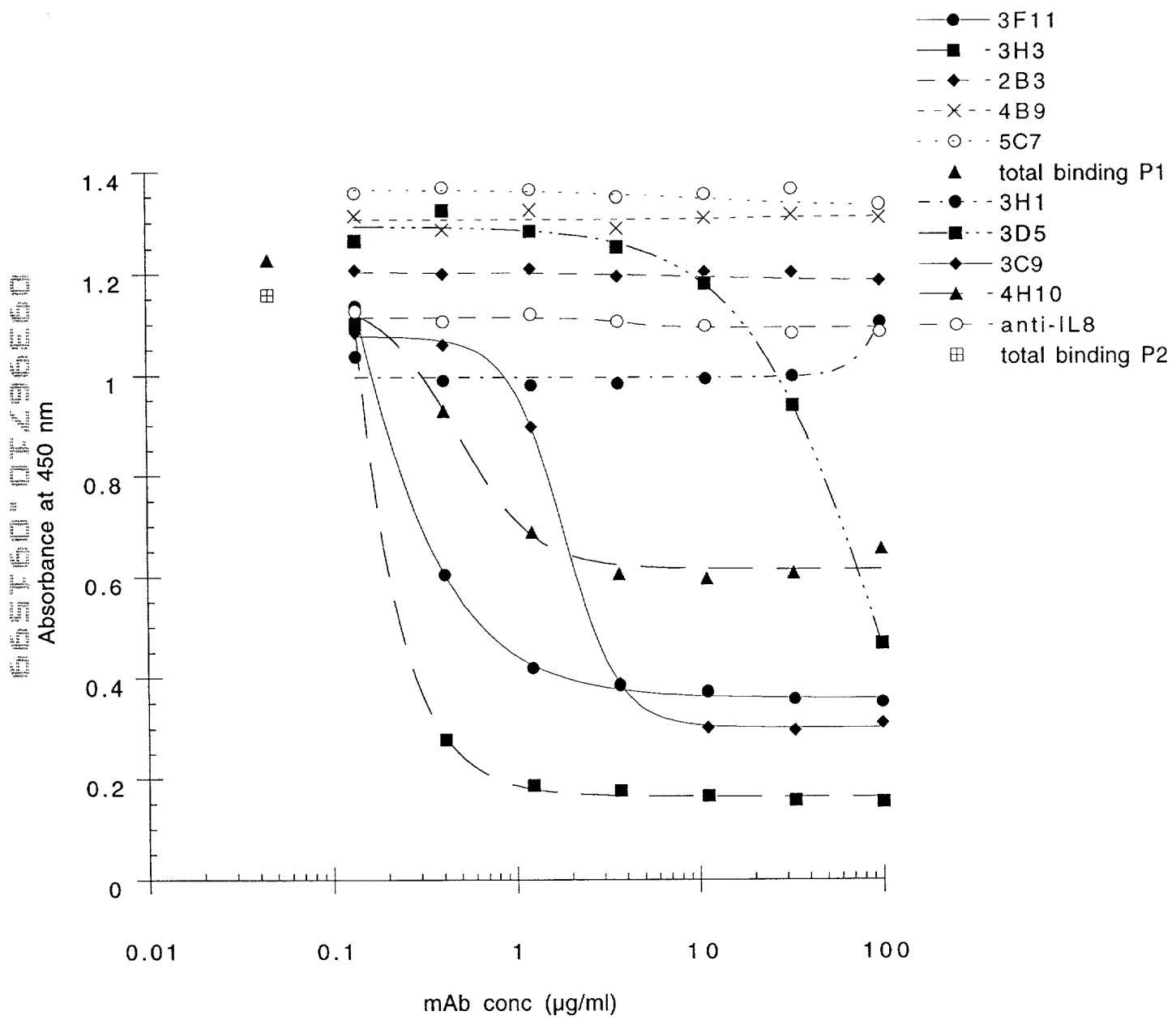
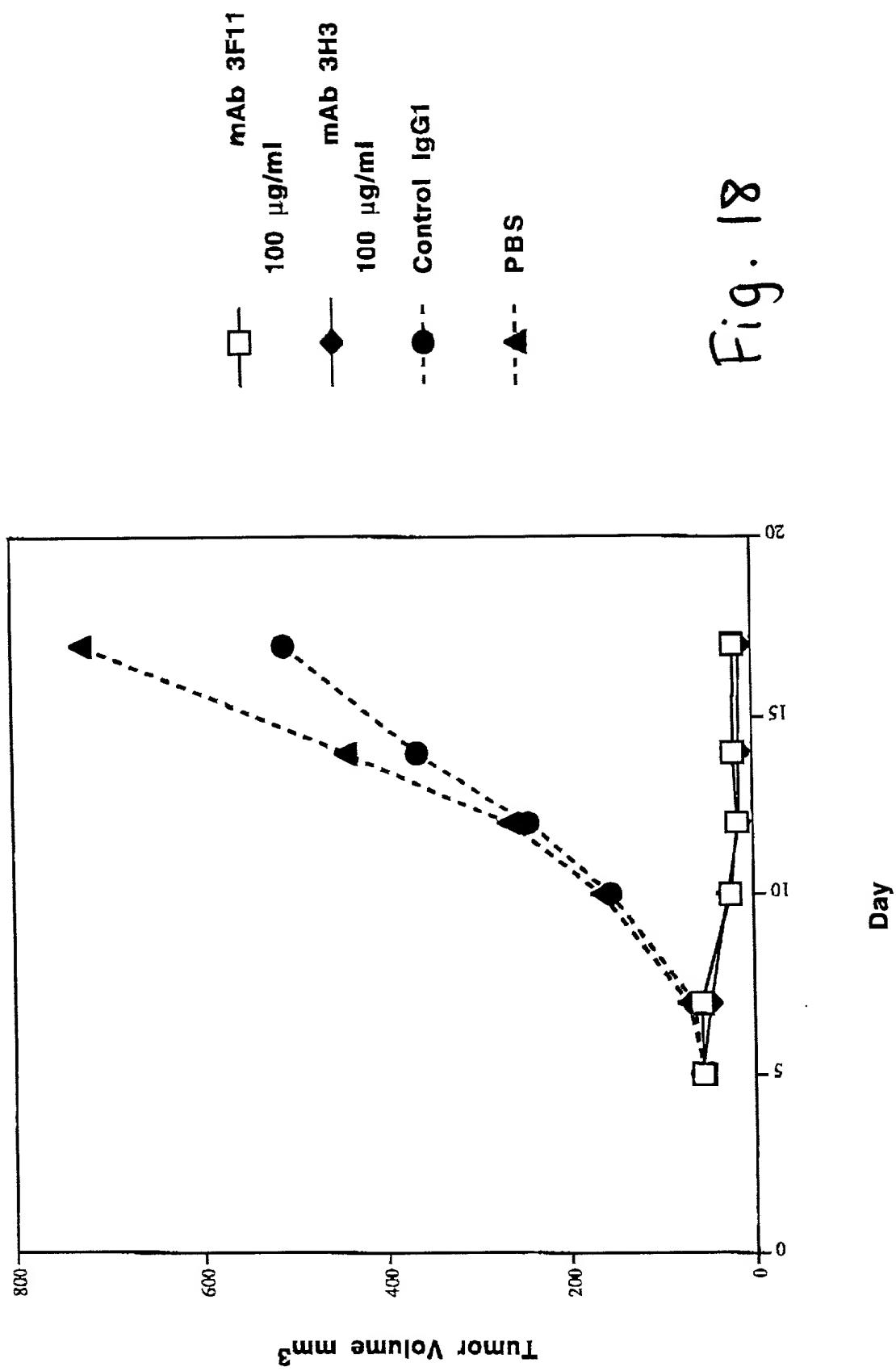


Fig. 17



Data Collection

	In-house	SSRL
Resolution (Å) ^b	30–3.5 (3.62–3.50) ^a	30–2.4 (2.49–2.40) ^a
R _{sym}	0.185 (0.398) ^a	0.056 (0.396) ^a
Number of observations	51,527	152,986
Unique reflections	12,459	38,908
Completeness (%)	99.8 (99.9) ^a	99.8 (99.7) ^a

Refinement

Resolution (Å)	30–2.4
Number of reflections	38,850
Final R ^c , R _{free} (F>0)	0.222, 0.267
Number of residues	781
Number of solvent molecules	286
Number of non-H atoms	6577
Average B factor (Å ²)	47.6
Rmsd bonds (Å)	0.013
Rmsd angles (°)	1.7
Rmsd B (bonded atoms) (Å ²)	2.4

^a Numbers in parentheses refer to the highest resolution shell.

^b $R_{sym} = \sum |I - \langle I \rangle| / \sum I$. $\langle I \rangle$ is the average intensity of symmetry related observations of a unique reflection.

^c $R = \sum |F_o - F_c| / \sum F_o$. R_{free} is calculated as R, but for 10% of the reflections excluded from all refinement.

Fig. 19

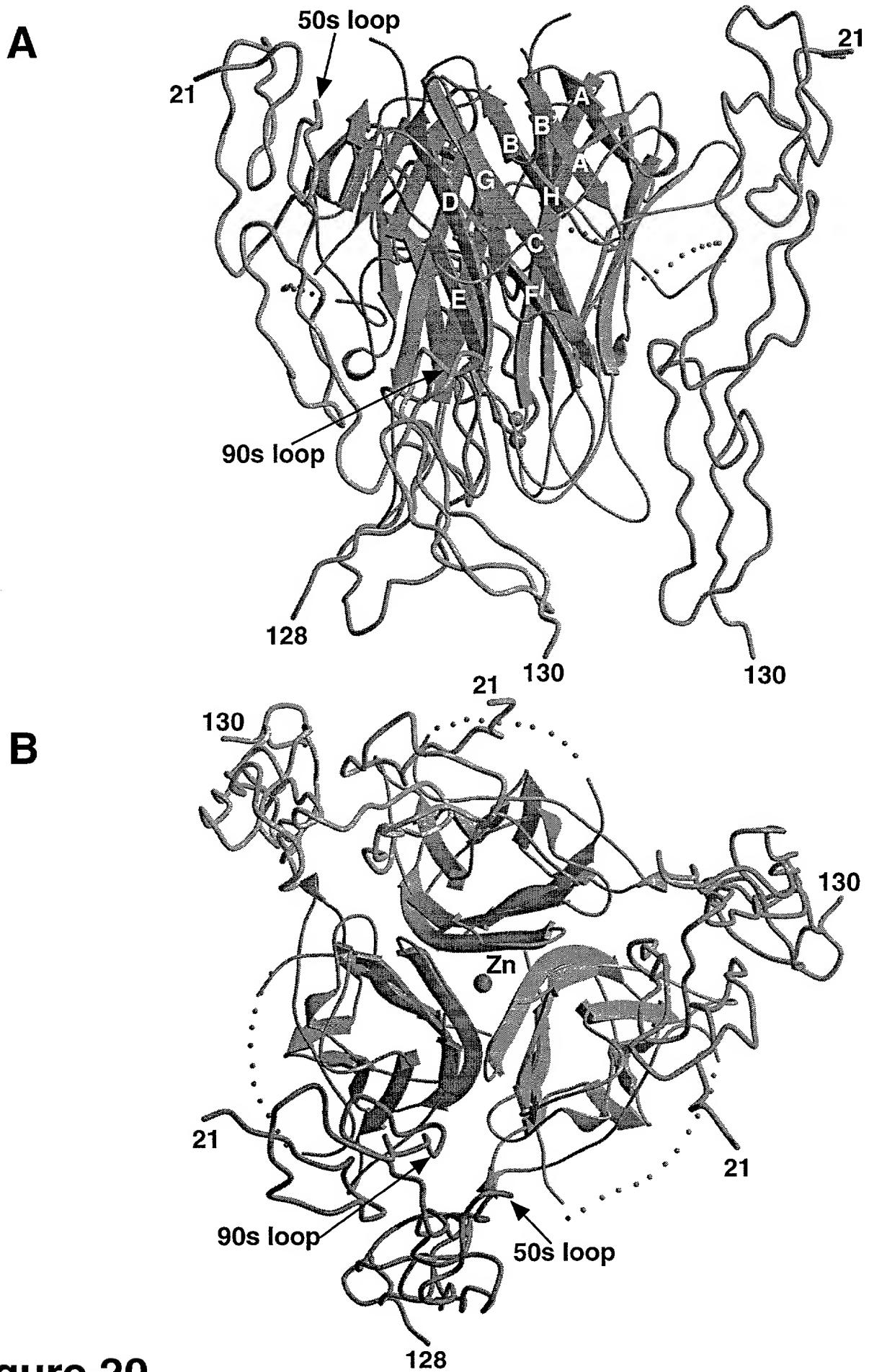


Figure 20

Figure 21

